ACETYLCHOLINE REDUCES MONOMERIC C-REACTIVE PROTEIN PRO-INFLAMMATORY ACTIVITY

R.S IEMMA

PhD 2021

ACETYLCHOLINE REDUCES MONOMERIC C-REACTIVE PROTEIN PRO-INFLAMMATORY ACTIVITY

ROCCO STEFANO IEMMA

A thesis submitted in partial fulfilment of the requirements of the Manchester Metropolitan University for the degree of Doctor of Philosophy

Department of Life Sciences
Manchester Metropolitan University
2021

SUMMARY

Acknowledgements

No PhD project can be done alone. This thesis is was done thanks to the assistance, supervision and moral support of several people. Therefore I would like to offer my sincere thanks to all of them.

There are many people that I must thank here and I apologise now, as I cannot mention everyone. During these three years I had assistance and help from so many people and I am grateful to each and every one of you. Most of you come from different parts of Europe and the World so I decided to thank you in your own languages.

I would like to express my sincere gratitude to my Director of Studies, Prof. Mark Slevin for support of my PhD study and research. My thanks also go to Mark Slevin's group (Glenn Ferris; Baoqiang Guo; Wei-Hui Fang; Donghui Liu; Vittorio Caprio). It has been an honour to work with all of you.

Ai miei genitori per il loro costante aiuto e continua preoccupazione nel cercar di non farmi mai mancare niente durante tutti i miei anni di studi universitari. A Flavia Iemma, per il supporto morale concessomi a distanza durante non solo questi tre ultimi anni di Dottorato ma anche in tutti gli anni di studi precedenti.

Dopo la mia famiglia biologica vorrei ringraziare il Dr. Sandro Satta per non solo perchè dall'alto della sua profonda conoscenza nel settore della biologia mi consigliato, suggerito e indirizzato in tutti quei momenti in cui la biologia ha mostrato il suo "Lato Oscuro", ma anche per l'enorme quantità di tempo e di pazienza che mi ha gentilmente dedicato nell'aiuto e nel supporto per la sottomissione di una tesi che possa essere all' altezza del titolo a cui aspiro.

Je voudrais remercie Pauline Gourisse. Je pense que la grandeur d'un homme dépend toujours de toutes les personnes qui l'entourent. Un projet de doctorat c'est très difficile de pouvoir l'accomplir. Je peux confirmer que je ne pourrais avoir fait ce que j'ai fait s'il n'y avait pas eu une personne comme Pauline Gourisse à mes côtés.

To Raya Karaganeva for the moral support received during this three long years even when the science showed its "Dark Side".

Quiero agradecer a Dani Moreno Martínez y Jonathan Gonzalez Sanchez por todas la risas y los chistes hechos en estos tres largos años necesario también a levantar el ánimo y el "pathos" para poder sobravivir a esta experiencia.

Je voudrais aussi remercier Mohamed El Mohtadi pour sa gentillesse, son aide, son soutien et son amitié démontrés pendant ces trois années.

Last but not least, Barbara Wade for her kidness and her patience in revising my thesis, and for reminding me constantly that I have to improve my English.

Published/ submitted works/ Honour and Awards

Publication

1. Slevin, Mark, **Rocco Iemma,** Yasmin Zeinolabediny, Donghui Liu, Glenn Robert Ferris, Vittorio Caprio, Mario Di Napoli, et al. 2018. "Acetylcholine Inhibits Monomeric C-Reactive Protein Induced Inflammation, Endothelial Cell Adhesion and Platelet Aggregation; A Potential Therapeutic?" Frontiers in Immunology 9 DOI: 10.3389/fimmu.2018.02124.

2. Caprio, V., Badimon, L., Di Napoli, M., Fang, W.-H., Ferris, G.R., Guo, B., **Iemma, R.S**., Liu, D., Zeinolabediny, Y., Slevin, M., 2018. pCRP-mCRP Dissociation Mechanisms as Potential Targets for the Development of Small-Molecule Anti-Inflammatory Chemotherapeutics. Front. Immunol. 9. DOI: 10.3389/fimmu.2018.01089.

Honours and Awards

1. 1° Place « Brain to Market » Summer School, Paris 3rd to the 7th of September 2018- Institut du Cerveau et de la Moelle Epinière (ICM)

2. 1° Place -Poster Presentation-9th International Conference on Dementia and Dementia Care 24-25 September 2018 London UK

Declaration

With the present declaration, I, Rocco Stefano IEMMA, born in Turin (Italy) on 30/10/1987, declare that this thesis and the work herein presented in it are my own, as well as the result of my original research investigations except for Figure 1.2 and 3.4 and the wester blot results in Figure 5.11 reported in chapter 5 obtained with the permission of Dr. Satta Sandro and Dr. Liu Donghui.

I hereby stated that this work has not been submitted, in whole or in part, in any previous application for a degree and is not currently being submitted in candidature for any degree other than the one of Doctor of Philosophy (PhD) at Manchester Metropolitan University (MMU).

Rocco Stefano IEMMA

Abbreviation List

Acetylcholine	ACh
Activator Protein-1	AP-1
Acute–Phase Response	APR
Adipose Tissue	AT
Alpha-1-acidglycoprotein	A1GP
Alzheimer's Disease	AD
Atherosclerotic Disease	ADs
Barthel Index	BI
Bicinchoninic Acid	BCA
Blood monocytes-derived-Macrophages	BMdM
Blood-Brain Barrier	BBB
Body Mass Index	BMI
Bovine Serum Albine	BSA
Brain-Derived Neurotrophic Factors	BDNF
C-Jun N-Terminal or Stress-Activated Protein Kinases	JNK/SAPK
C-Reactive Protein	CRP
Cardiovascular Disease	CVD
Cell Adhesion Molecules	CAMs
Ceruloplasmin	Cp
Cholinergic Anti-Inflammatory Pathway	CAIP
Common Myeloid Progenitor	CMP
complementary Deoxyribo Nucleic Acid	cDNA
Confidence Intervals	CI
Damage Associated Molecular Pattern Molecules	DAMPs
Dendritic Cells	DCs
Deoxyribo Nucleic Acid	DNA
Diabetes Mellitus	DM
Dimethyl sulfoxide	DMSO
Dulbecco's Phosphate Buffered Saline	DPBS
Endothelial Cells	ECs
Enzyme-Linked Immunosorbent Assay	ELISA
ERK/ Big MAP Kinase 1	BMK1
Erythrocyte Sedimentation Rate	ESR
Extracellular Signal-Regulated Kinases	ERKs
Fetal Bovine Serum	FBS
Florescence-Activated Cell Sorting	FACS
Fluorescein isothiocyanate	FITC
Granulocyte-Macrophages Progenitor	GMP
Haptoglobin,	Hp
Haematopoietic Stem Cell	HSC
High-Mobility Group Binding Protein 1	HMGB1
High-Mobility Group Binding Protein 1 HMGB-1	HMGB-1
Hypoxia-inducible factor 1-α	HIF1-α
Industrial Methylated Spirit	IMS
Insulin Growth Factor-1	IGF-1
Intercellular adhesion molecule-1	ICAM-1
Intercellular Adhesn Molecule-1	ICAM-1
Interlukin	IL
Intracerebral Haemorrhage	ICH
Ischemic Stroke	IS
Lipopolysaccharides	LPS
Lysophosphatidylcholine	LPC
Metabolic Syndrome	MetS

Methyllycaconitine Citrate	MLA
Middle Cerebral Artery Occlusion	MCAO
Mitogen-Activated Protein Kinase	MAPK
Mitogen-Activated Protein Kinases p38	MAPKp38
Modified Rankin Scale	mRS
Monocyte Chemoattractant Protein-1	MCP-1
Monocyte Chemoattractant protein-1	MCP-1
Monocyte chemoattractant protein-1	MCP-1
Monocyte Count	MC
Monomeric C-Reactive Protein	mCRP
Mononuclear Phagocyte System	MPS
Neutrophils Cells	NCs
Nitric Oxide	NO
Nitric Oxide Synthase	iNOS
Noncommunicable chronic diseases	NCDs
Nuclear Factors KB	NF-KB
Obese-ype 2 diabetes mellitus	OT2DM
Phorbol-12-myristate 13-acetate	PMA
Phosphate Buffered Saline	PBS
Plasminogen activator inhibitor 1	PAI-1
Polymerase Chain Reaction	PCR
Post-Stroke Cognitive Impairment	PSCI
Propidium Iodide	PI
Radioimmunoprecipitation	RIPA
Reactive Oxygen Species	ROS
Real-Time Polymerase Chain Reaction	RT-qPCR
Retinol Binding Protein	RBP
Reverse transcriptases-qPCR	RT-PCR
Ribonucleic Acid	RNA
Serum Amyloid A	SAA
Signal Transducer and Activator of Transcription-3	STAT3
Small Molecular Inhibitors	SMIs
Tight Junctions	TJs
Tissue Resident Macrophages	TRM
Toll-Like Receptors	TRL
Transcription Factor	TF
Transforming Growth Factor Beta	TGF-β
Tumor Necrosis Factor-α	TNF-α
Vagus Nerve	VN
Vascular Adhesion Molecule-1	VCAM-1
Vascular cell adhesion protein 1	VCAM-1
Vascular Endothelial Growth Factor	VEGF
Western Blotting	WB
White Blood Cell	WBC
Wound Healing Process	WHP
α7 nicotinic Acetylcholine Receptor	α7nAChR

List of Figure

CHAPTER 5

CHAPTER 6

List of Table

Abstract

INTRODUCTION: inflammation increases the production of pro-inflammatory cytokines, that, on binding to their respective receptors in the liver, stimulate the synthesis of native CRP (nCRP). nCRP can be dissociated into sub-units or monomeric CRPs (mCRPs) and can be locally accumulated with macrophages in the same chronic inflamed tissue. Therefore, the goal of the present project was to investigate the potential of mCRP pro-inflammatory activity and the potential of new effective small molecule inhibitors (SMIs) in blocking mCRP effects. **MATERIALS AND METHODS:** differentiated human U937 macrophage-like cells were stimulated by mCRP in the presence or absence of small molecular compounds, such as Acetylcholine (ACh) and Nicotine or the 3H12 antibody. The levels of tumour necrosis factor-α (TNF-α), Interleukin-6 (IL-6); and IL-10 were determined by enzyme-linked immunosorbent (ELISA) assay. Western Blotting was used to evaluate the intrinsic pro-inflammatory pathway. The RT-PCR was used to determine mCRP RNA gene expression. **RESULTS:** mCRP increased TNF-α and IL-6 concentrations. ACh significantly inhibited the mCRP induced TNF-α and IL-6 release. Western blotting confirmed that ACh blocks mCRP-induced cell signalling phosphorylation of ERK, JNK, and NF-KB. The RT-PCR results showed that mCRP increases cytokine RNA expression, whereas ACh was unable to reduce it. **CONCLUSIONS:** this study demonstrated that mCRP had a robust pro-inflammatory activity, whilst the use of ACh inhibited its pro-inflammatory activity, therefore reducing TNF-α, IL-6 and IL-10 release. Indeed, it is likely that the monomeric CRP could be a promising new target for the prevention and treatment of chronic inflammation tissue associated with chronic kidney diseases, arteriosclerosis and stroke.

Keywords: Inflammation, monomeric C-reactive protein; inflammation; macrophage, ACh, Nicotine

CHAPTER 1
INTRODUCTION.

1.1.1 Inflammation.

Injury can interfere with the fundamental equilibrium (homeostasis) necessary for the survival of the organism. After homeostasis disruption, inflammation steps in as the first phase of the wound healing process (WHP) (Serra et al., 2017). The word inflammation comes from the Latin term, “inflammare” (to set on fire) and was first designated by Cornelius Celsus, who described this phenomenon using adjectives such as swelling, redness, heat, and pain (Freire and Dyke, 2013). Inflammation is a natural mechanism, with the primary aim of protecting the host from any factors that could potentially interfere with normal homeostasis (Chen et al., 2018). A brief summary of the inflammation phase will be given for the purpose of the studies described in this thesis.

1.1.2 Acute Inflammation.

The most commonly example used to explain the inflammation phase is that of Acute Skin Wound Healing (Eming et al., 2007; Larouche et al., 2018; Canedo-Dorantes and Canedo-Ayala, 2019). This process starts when the components of the coagulation cascade and immune system cells are attracted to the injured site to avoid substantial blood and fluid loss (Weiss, 2008). This mechanism is triggered to prevent potential and hazardous infections and to eliminate dead tissues (Gurtner et al., 2008). That is, the first bacterial pathogens to penetrate the injury are identified by the Toll-like receptors (TLRs), which are trans-membrane-spanning receptors normally expressed in sentinel cells, such as tissue resident macrophages, dendritic cells, neutrophils and monocytes (Gerold et al., 2007; Hemmi and Akira, 2005; Prince et al., 2011). Once the pathogens have been recognized, this process allows their elimination through the production of pro-inflammatory cytokines, chemokines and prostaglandins (Freire and Dyke, 2013). At the beginning of the inflammatory stage, endothelial cells (ECs) increase the expression of both chemokines and cell adhesion molecules (CAMs) on the surface of their membrane (Martin, 1997; P et al., 2003). Both chemokines and CAMs allow for the recruitment of neutrophils from the circulation to the wound site (Pate et al., 2010). Once recruited, neutrophils eliminate invading agents and/or other potentially hazardous microorganisms, by increasing the concentration

of chemokines, cytokines and active antimicrobial substances, such as reactive oxygen species (ROS) (Gonzalez et al., 2016). The exponential increase of several chemotactic cytokines (Deshmane et al., 2009) is followed by an invasion of mononuclear phagocyte cells (monocytes) through vascular leakage (Rodero et al., 2014). Once monocytes have differentiated into macrophages (Yang et al., 2014a), they increase the concentration of several pro-inflammatory cytokines and eliminate any potentially harmful microbicidal activity (Thuraisingam et al., 2010; Rodero and Khosrotehrani, 2010).

1.1.3 Cytokines.

Cytokines are specifically secreted proteins, such as lymphokines, monokines, chemokines and interleukins with autocrine, paracrine and/or endocrine activity (Zhang and An, 2007). These proteins are released by several cells during the inflammatory response to activate and regulate the immune system response (Kany et al., 2019).

1.1.3.1 Tumor Necrosis Factor-α (TNF-α).

TNF-α is a pleotrophic polypeptide, which plays a pivotal role during inflammation, cell proliferation, differentiation and apoptotic activities (Feuerstein et al., 1994; Baud and Karin, 2001). This 26 kDa homotrimer transmembrane protein is synthesised by several immune system cells (T-lymphocytes, natural killer cells and macrophages) when they are stimulated by Lipopolysaccharides (LPS) or in the presence of other pathological medical conditions (Van Deventer, 1997; van der Bruggen et al., 1999; Zelová and Hošek, 2013; Bokhari et al., 2014). After being synthesised, TNF-α remains attached to the cell surface until the TNF-converting enzyme modifies it into a soluble TNF form with a molecular mass of 17 kDa (Ramana, 2010). Once converted, this pro-inflammatory cytokine is released into the bloodstream (Black et al., 1997). Under normal physiological conditions, there is a low TNF-α level (Mirzaei and Mahmoudi, 2018). However, during infection or traumatic events, there is a significant rise in the TNF-α concentration (Robak et al., 1998; B. Johnston and Conly, 2006). When TNF is released into the

bloodstream, this pro-inflammatory cytokine mediates its activity through the activation of TNF receptors (TNFR1 or TNFR2) that are expressed in all tissues (Yang et al., 2018). Both TNFR1 and/or TNFR2 are capable of stimulating the nuclear factor kappa-light-chain-enhancer of activated B cells (also known as a Nuclear Factor-KB or NF-KB) which leads to a cell death (TNFR1) or cell survival (TNFR2) response (Yang et al., 2018). Once TNF-α is released, together with interleukin-6 (IL-6), it triggers several acute-phase protein (APPs) secretions (Moshage, 1997), such as the pentameric or native C-reactin protein (nCRP) and or alpha 1-acid glycoprotein (alpha 1AG) (Meijer et al., 1993). Evidence of the capacity TNF-α has to increase nCRP production has been reported by Calabro et al. (2003) who observed that there is an increase in nCRP synthesis when human coronary artery smooth muscle cells (HCASMCs) are exposed to TNF-α.

1.1.3.2 Interleukin-1β (IL-1β).

IL-1β is a pro-inflammatory cytokine belonging to the IL-1 family (Allan et al., 2005). IL-1β is secreted when extra cellular stimuli, such as Damage Associated Molecular Pattern molecules (DAMPs), Pathogen Associated Molecular Pattern (PAMPs) viruses, LPS, UV light, oxidative stresses, inflammatory stimuli and cytokines (Shih et al., 2015), bind to pattern recognition receptors (PRRs) (Lopez-Castejon and Brough, 2011). When PRRs activate the mitogen-activated protein kinase (MAPK) this allows NF-KB to translocate into the nucleus and to up-regulate the IL-1β gene expression (de Oliveira et al., 2017). The up-regulation of the IL-1β gene expression increases the synthesis and release of IL-1β pro-inflammatory cytokines, which, once released into the circulation, bind to the IL-1 receptor (IL-1R) (Jensen et al., 2000; Meier et al., 2019). When IL-1β and IL-6 bind to their respective receptors on hepatocyte cells, these two pro-inflammatory cytokines trigger the release of several APPs, such as nCRP (Szalai et al., 1998b; Calabro et al., 2003; Kramer et al., 2008). This process triggers Acute Phase Response (APR) activity (Dinarello, 2009; Dinarello et al., 2012; Garlanda et al., 2013).

1.1.3.3 Interleukin-6 (IL-6).

IL-6 is a pro-inflammatory mediator which was first discovered in 1986 as a B-cell differentiation factor-2 (BSF-2) (Hirano et al., 1986). This pro-inflammatory cytokine is secreted by several cells, such as ECs, neurons and macrophages (Chi et al., 2001; Li et al., 2009; Erta et al., 2012). IL-6 synthesis occurs when viruses, LPS, UV light, oxidative stresses, inflammatory stimuli or cytokines (Shih et al., 2015) bind to the PRRs (Pires et al., 2018). This allows the NF-KB to translocate into the nucleus and up-regulate the IL-6 mRNA gene expression (Lawrence, 2009; Tanaka et al., 2014). Once released into the bloodstream, IL-6 binds to hepatocyte cells (Schmidt-Arras and Rose-John, 2016). This bond allows the CCAAT/enhancer binding protein β (C/EBPβ) and the C/EBPδ transcription factors to enhance the amount of nCRP released into the bloodstream (Kushner and Feldmann, 1978; Heinrich et al., 1990; Zhang et al., 1996; Szalai et al., 1998a; Du Clos and Mold, 2004). Literature has reported a strong correlation between the amount of IL-6 released during inflammation activity and the increase in nCRP concentration (Szalai et al., 1998a). A linear correlation between elevated IL-6 and nCRP levels has been confirmed in several patients with different types of infections (Sahbudak Bal et al., 2017; Herold et al., 2020) or medical diseases such as obesity, cancer, chronic kidney disease (CKD), atherosclerosis and stroke (Khaodhiar et al., 2004; Béténé A Dooko et al., 2014; Luna et al., 2014).

1.1.4 Acute-phase response (APR).

When the inflammation phase increases the pro-inflammatory cytokine release, these pro-inflammatory markers bind to hepatocyte cells (Szalai et al., 1998b; Calabro et al., 2003; Kramer et al., 2008; Schmidt-Arras and Rose-John, 2016) and stimulate the liver to produce APPs (**Fig 1.1**) which are released into the circulation (Zhang and An, 2007; Zhou et al., 2016). Amongst the several pro-inflammatory cytokines, IL-6 has been identified as a the major stimulator of numerous APP proteins (Heinrich et al., 1990). When IL-6 binds to the IL-6R present on hepatocyte cells (Tanaka et al., 2014), this pro-inflammatory cytokine activates the signal transducer and activator of

transcription-3 (STAT3) factor, C/EBP transcription factors and the NF-KB the transcription factors (Agrawal et al., 2003). The activation of these intrinsic pathways regulates the transcription of APR genes (Lowenstein and Matsushita, 2004). Activation of APR genes decreases the transthyretin, retinol binding protein (RBP), albumin and transferrin circulation protein but increases the nCRP, serum amyloid A (SAA), haptoglobin (Hp), alpha-1-acidglycoprotein (A1GP), ceruloplasmin (Cp) and fibrinogen blood concentration (Ceron et al., 2005; Khalil and Al-Humadi, 2020). The increase in the aforementioned APPs are categorised as major when the APP increase is 10- to 100-fold, moderate when the APP increase is 2- to 10-fold, or minor when the APP increase is minimal (< 2-fold) (Cray et al., 2009). When there is a high concentration of both pro-inflammatory cytokines and APPs in blood, a wide range of biological, physiological, metabolic, immunological and neuro-endocrinal changes take place (Gruys et al., 2005; Jain et al., 2011). This response is also known as an APR (Cray et al., 2009). Both inflammation and APR are identified by the presence of some pro-inflammatory mediators, such as nCRP (Jain et al., 2011; Ahmed et al., 2012).

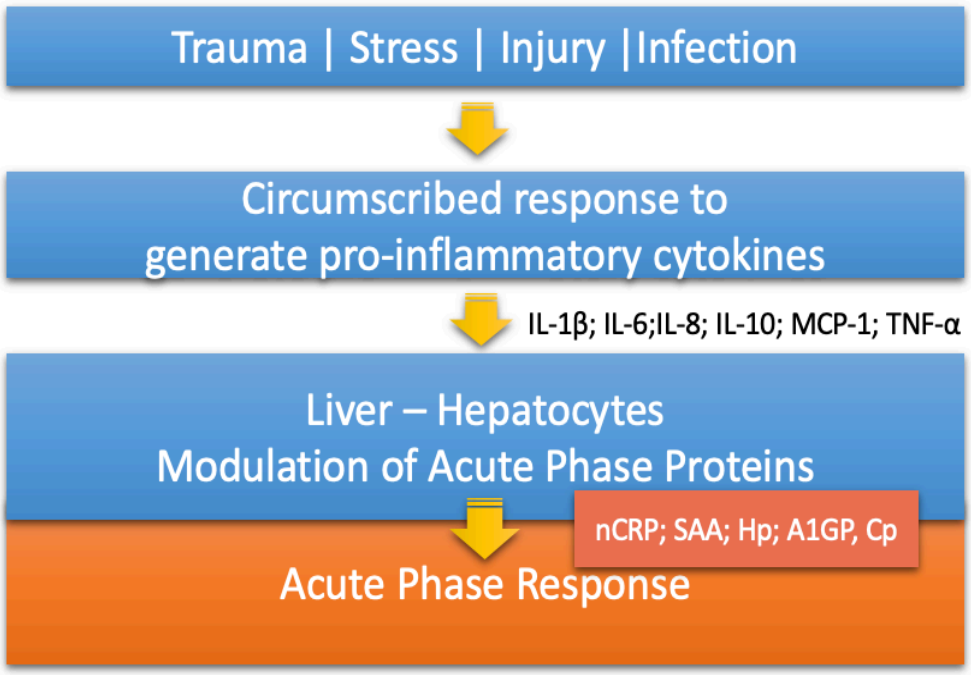


Fig 1.1. Acute-phase response. A schematic summary of how trauma, stress, injury and/or infection trigger the acute phase response. These factors increase the production of pro-inflammatory cytokines, that, on binding to their respective receptor in the liver, they induce the synthesis of several APPs (nCRP has been recognised as a one of the most important). Adapted from: Cray, C., Zaias, J. and Altman, N. H. (2009) 'Acute phase response in animals: a review.' Comp Med, 59(6), Dec, 2009/12/26, pp. 517-526.

1.1.5 The pentameric or native C-reactive protein (nCRP).

Particular attention has been paid to the study of nCRP, due to its capacity to rise exponentially in reaction to both infection and/or tissue damage (Gans et al., 2015). Various cell types can synthesize and secrete nCRP, such as peripheral blood lymphocytes (Kuta and Baum, 1986), respiratory epithelium (Sattar et al., 1999), neurons (Yasojima et al., 2000) , endothelial cells (Pasceri et al., 2000a), smooth muscle cells (Calabro et al., 2003), macrophages (Devaraj et al., 2009) and mainly hepatocytes (Hurlimann et al., 1966). The monomer of this protein has a molecular weight of 23 kDa (Osmand et al., 1977) and composed of 206 amino acids aligned into two β-sheets (Shrive et al., 1996). Once synthesised, the monomers are assembled through disulfide bonds (Goodman et al., 1996). This process occurs in the endoplasmic reticulum and allows the nCRP to take on a pentameric form (**Fig 1.2**). Pentameric CRP is made up of five noncovalently associated protomers equally positionated around a central hole (Thompson et al., 1999; Du Clos and Mold, 2004). The pentameric or native-CRP (nCRP) forms remain inside the endoplasmic reticulum until the pro-inflammatory cytokine IL-6 (Szalai et al., 1998a; Du Clos and Mold, 2004) binds to the hepatocyte cells (Kushner and Feldmann, 1978; Zhang et al., 1996; Szalai et al., 1998a; Du Clos and Mold, 2004).

Increased levels of nCRP have been reported in several types of infective mechanisms. Indeed, Póvoa et al. (2005) observed a substantial rise in the nCRP concentration (>8.7 mg/dL) in subjects with fungal infection with Gram-positive and Gram-negative bacteria. Liu et al. (2010) reported that nCRP is a useful clinical biological marker, predictive of potential bacterial infection in the aging population. Haran et al. (2013) reported that there is also a rise in the nCRP protein (135.96 mg/L) in those affected by influenza and/or by a bacterial infection.

nCRP is released into the circulation to discern pathogenic agents from damaged cells (Volanakis, 2001). Indeed, Ca^{2+}-dependent binding enables

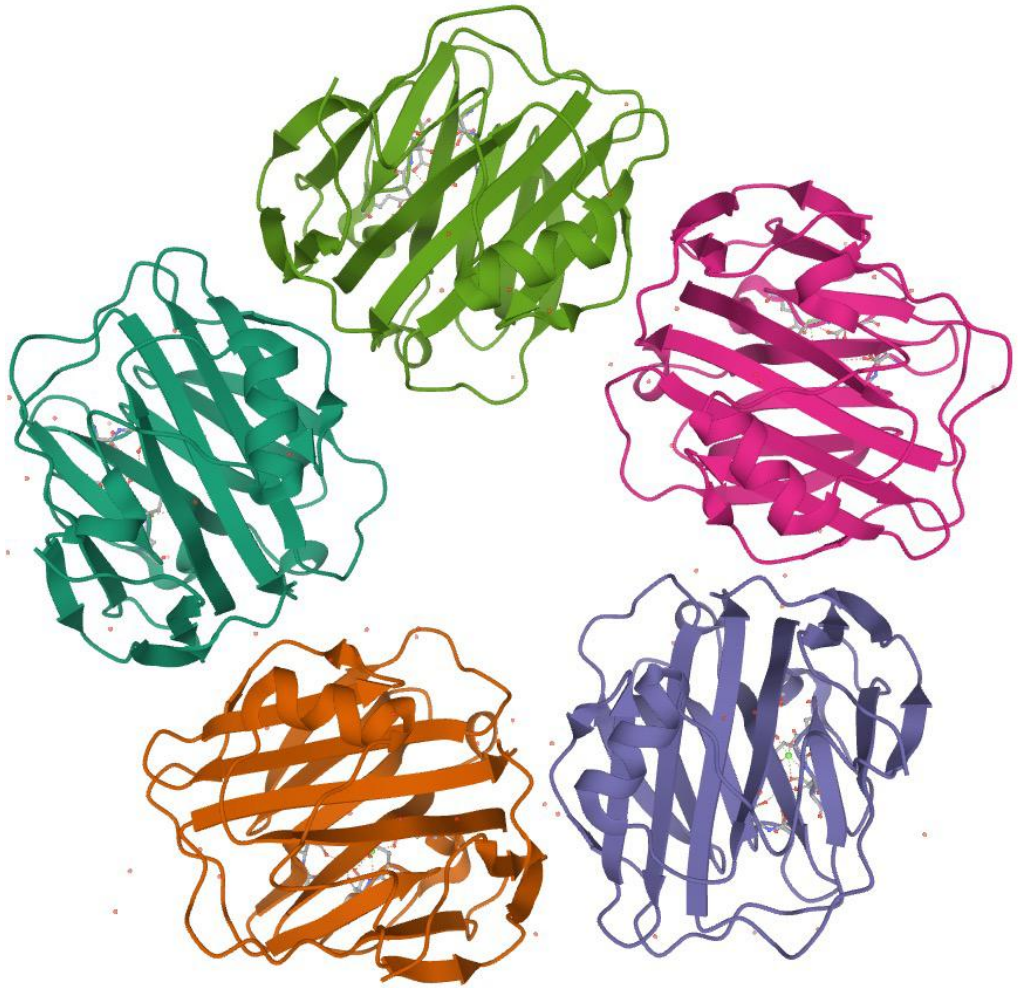


Fig 1.2. **The pentameric or native C- reactive protein.** This figure shows the native C- reactive protein (nCRP) in its typical annular pentameric form.

each monomer to bind to the phosphocholine (PCh) expressed in a lot of bacteria and/or fungus (Volanakis and Kaplan, 1971; Boncler and Watala, 2009). When this binding occurs, nCRP triggers both the complement system and the phagocytosis activity which allows for the identification and elimination of bacteria (Mortensen, 2001; Chaplin, 2010). For example, an *in vivo* study reported that nCRP has a protective capacity when it binds to the PCh present in gram-positive pathogens such as *Streptococcus pneumoniae* through the activation of the complement system (Mold et al., 1981), Szalai et al. (2000) also reported that nCRP had protective proprieties against *Salmonella enterica serovar typhimurium* infection in transgenic CRPtg C57BL/6J congenic mice. Once the defensive activity of nCRP is concluded, this pentatrix is removed to the circulation and catabolized by hepatocytes (Hutchinson et al., 1994; Markanday, 2015).

1.1.6 Chronic Inflammation.

Under normal physiological conditions, a reduction in the level of nCRP is a sign that acute inflammation is progressing to the following stage (Kasapis and Thompson, 2005; North et al., 2009). However, sometimes the acute inflammation process does not progress and there is a continuous neutrophil and monocyte/macrophage recruitment (Motwani and Gilroy, 2015) alongside a substantial and consistent presence of: *(I)* several pro-inflammatory mediators (Histamine, Eicosanoids); *(II)* pro-inflammatory cytokines (TNF-α, IL-1β, IL-6); *(III)* and APPs, such as nCRP (Lawrence and Gilroy, 2007; Luan and Yao, 2018; Slevin and Molins, 2018). This chronic inflammation activity leads to tissue injury (Franceschi and Campisi, 2014) and is often associated with several noncommunicable chronic diseases (NCDs) (Camps and García-Heredia, 2014) such as obesity, diabetes, cancer, Chronic Kidney Disease (CKD), Alzheimer's disease (AD) and cardiovascular disease (CVD) (Akiyama et al., 2000; Kovesdy et al., 2017; Gupta et al., 2018).

1.1.7 nCRP and its association with noncommunicable chronic diseases (NCDs).

As aforementioned in paragraphs 1.1.5 and 1.1.6, nCRP is a biological marker useful to indicate the potential ongoing event of acute infection and/or chronic inflammation. Indeed, the last two decades have witnessed the publication of studies that have reported an association between higher nCRP concentrations (>3μg/mL) and an increased risk of several NCDs, such as obesity, diabetes, cancer, stroke, atherosclerosis and AD (Black et al., 2004; Kushner et al., 2006; Luan and Yao, 2018).

1.1.7.1 nCRP and Obesity.

Obesity is an abnormal or excessive adipose tissue (AT) accumulation (Palou and Bonet, 2013). Under normal physiological conditions, the main role of AT is to store excess nutrients (Zwick et al., 2018). However, in the case of obesity, AT releases pro-inflammatory mediators, such as the monocyte chemoattractant protein-1 (MCP-1), the plasminogen activator inhibitor 1 (PAI-1), the high-mobility group binding protein 1 (HMGB-1), angiotensin II, TNF-α, IL-6 and leptin (Makki et al., 2013; Guzmán-Ruiz et al., 2014). Both IL-6 and leptin are present in the circulation of obese people (Eder et al., 2009; DePaoli, 2014). These two mediators bind to hepatocytes and stimulate both nCRP synthesis and release (Szalai et al., 1998a; Du Clos and Mold, 2004; Loffreda et al., 1998). A study by Visser et al. (1999) reported that young people with a high body mass index (BMI) had a high concentration of several pro-inflammatory markers and nCRP (>0.22 mg/dL) (Visser et al., 1999). Another study reported a strong association between obesity, high nCRP levels (>10 mg/L) and metabolic syndromes (MetS) (Aronson et al., 2004). Kahn et al. (2006) published similar results, confirming that obesity is the main factor associated to nCRP and MetS. Investigation on 493 obese patients, with a BMI from 31 to 91 kg/m^2, showed that those with higher nCRP levels (>7.7 mg/L) ran a higher risk of suffering from depression (Dixon et al., 2008). Choi et al. (2013) reported that higher nCRP levels were associated to obesity, in particular in females. Paepegaey et al. (2015) reported on 674 obese patients

with a BMI from 47.4 ± 7.4 kg/m^2 and stated that there was a higher nCRP level, especially in females. Elevated BMI levels, nCRP and HMGB-1 were also reported in a study by Guzmán-Ruiz et al. (2014).

1.1.7.2 nCRP and Diabetes.

Literature reports that there is an elevated serum HMGB-1 concentration in obese populations (Gunasekaran et al., 2013). When circulating HMGB-1 dissociates into a HMGB1 biological active isoform, it is able to penetrate into the bone marrow and stimulate the release of monocytes into the circulation (Liesz et al., 2015). This release accounts for findings that there is a rise in the white blood cell (WBC) count in obese-type 2 diabetes mellitus (OT2DM) patients (Colberg et al., 2010; Gkrania-Klotsas et al., 2010; Milam, 2016; Colberg et al., 2016; Zhou et al., 2018). Once in the circulation, both monocytes and macropahges bind to the highly concentrated blood glucose (Al-Nuaim, 1997; Lafontan, 2005; Daniele et al., 2014; Malin et al., 2018). This binding activates both the aerobic glycolysis metabolism and the hypoxia-inducible factor 1-α (HIF1-α) transcription factor, increasing the release of several pro-inflammatory cytokines (Galván-Peña and O'Neill, 2014). If this release continues, it leads to a systemic chronic low-grade inflammation (Tsalamandris et al., 2019). This systemic chronic low-grade inflammation then stimulates the release of nCRP (Ziv-Baran et al., 2017). A study reported that elevated fibrinogen, IL-6 and nCRP levels were associated with the risk of developing diabetes (Bertoni et al., 2010). Wang et al. (2013) studied 4,213 Japanese subjects, age range 35 to 66 and stated that elevated nCRP (>10 mg/L) levels preceded the onset of T2DM and that this result was not affected by their being either obese or smokers. de Rekeneire et al. (2006) reported elevated nCRP (34.5 mg/L) concentrations in subjects affected by diabetes. Alam et al. (2014) reported that elevated nCRP levels (5.290 mg/L) were also associated to high ferritin levels in 67 diabetic patients. El Boukhrissi et al. (2017) stated that significantly high nCRP levels (>5.94 mg/L) were also confirmed in 250 T2DM patients with a BMI from 27.2 ± 6.8 kg/m^2. Furthermore, several studies have also reported that high nCRP concentrations in diabetic subjects were also associated to the risk of

developing other diabetic complications and/or diabetes-related diseases. Indeed, elevated nCRP concentrations have been associated with the risk of developing atherosclerosis (Mugabo et al., 2010). In a Japanese study on 3,573 T2DM patients, aged 62 to 64 years, with a BMI from 24.6 ± 2 kg/m^2 it was reported that high levels of nCRP were also associated with a higher incidence of depression (Hayashino et al., 2014). A recent study reported an association between nCRP levels and CVD in 40 diabetic patients (Ahmed et al., 2020). More recently, it has also been reported that elevated nCRP levels in OT2DM were associated with a higher risk of developing cancer (Lee et al., 2011; Kolb and Zhang, 2020).

1.1.7.3 nCRP and Cancer.

Recently, some authors have reported that the low-chronic grade of inflammation caused by both obesity and diabetes also increases the risk of developing cancer (Lega and Lipscombe, 2020). The presence of nCRP has been observed in cancer (Lee et al., 2011; Shimura et al., 2012; Shrotriya et al., 2018). Indeed, Yang et al. (2007) reported that when nCRP binds to myeloma cells through FC-γ receptors (FCγRs), this pentameric protein phosphorylates the Akt, ERK and NF-KBp65 pathways and, in turn annuls the effects of therapeutic compounds, such as dexamethasone and melphalan. Paulsen et al. (2017) detected elevated IL-6 and nCRP levels in 49 advanced cancer patients. Agnoli et al. (2017) reported that high nCRP levels (1150 ng/mL) were also associated to a higher risk of breast cancer (BC) in postmenopausal women. Pastorino et al. (2017) stated that higher nCRP concentration could be a predictor of immediate and/or long-term mortality in lung cancer (LC). Fang et al. (2017) on nasopharyngeal carcinoma reported that there was a linear association between nCRP concentration (≥1.96 mg/L) and a worse prognosis and higher mortality.

1.1.7.4 nCRP and Stroke.

A 2006 study reported that an obesity-associated low-chronic level of inflammation, also creates a predisposition for the development of CVD

(Poirier et al., 2006). Research, published in the New England Journal of Medicine evidenced the presence of higher baseline levels of serum amyloid A, sICAM-1, IL-6, total cholesterol, apolipoprotein B-100 and in particular nCRP and their association with cardiovascular accidents, such as stroke (Ridker et al., 2000).

Stroke is a neurological medical condition of ischemic or haemorrhagic nature (intracerebral haemorrhage –ICH-, subarachnoid haemorrhage) due to a critical focal injury to the brain (Sacco et al., 2013). This neurological medical condition can induce long-term disability, or even death (Starosta et al., 2016; Ågesen et al., 2018). In 2011 an AHA report (Roger et al., 2011) confirmed that, of ≈610,000 people yearly suffering from a first stroke attack, ≈185,000 had recurrent attacks, while of the remaining patients (≈425,000 people) 87% were affected by an ischemic stroke (IS) event and 13% by a haemorrhagic stroke event.

Considering the strong correlation between nCRP and stroke one may speculate that this neurological medical condition could be predicted by evaluating the baseline nCRP levels (den Hertog et al., 2009). A study by Rost et al. (2001) reported that an excessive nCRP concentration (>5.4 ± 7.3 μg/mL) was associated to a higher risk of stroke in both genders, with a higher frequency in women. Similar results were also reported in another study, where nCRP and ICAM-1 serum levels were predictive of a higher stroke risk (Jiménez et al., 2016). Even a single stroke symptom, such as unilateral numbness, unilateral weakness and disability to communicate verbally or in writing form, was recently found to be correlated to anomalous levels of nCRP, NT-pro BNP, Factor VIII, Factor XI and D-dimer (Landry et al., 2017).

Extensive studies have been carried out on ischemic stroke (IS) and its correlation with nCRP (Di Napoli et al., 2001b; Di Napoli et al., 2001a; Winbeck et al., 2002; den Hertog et al., 2009; Bakhshayesh-Eghbali et al., 2016). IS is characterised by vessel occlusion as a consequence of cardioembolic, atherothrombotic and/or lacunar events (Meschia and Brott, 2018). The occlusion leads to a significant decrease in cerebral blood flow (CBF)

associated with a cascade of biological activity and neural cell death (Meschia and Brott, 2018). It was observed that ROS generation (from the *penumbra area*) stimulated the surviving neurons to release cytokines and chemokines to activate the tissue-resident macrophage (TRM) microglia (Wang et al., 2006; Qin et al., 2019). Once activated, microglia increase the level of several pro-inflammatory mediators, such as iNOS, IL-1β MCP-1, IL-6 and TNF-α (Gelosa et al., 2014; Zarruk et al., 2018). Therefore, the rapid and unexpected increase in this pro-inflammatory mediator, acting on TJs proteins (occluding, ZO-1, ZO-2 and claudin-5), lead to both TJs phosphorylation, destabilisation and nullification (Dörfel and Huber, 2012), which, in turn, facilitates blood brain barrier (BBB) disruption within the first 3 hours of IS onset (Dörfel and Huber, 2012; Giraud et al., 2015). This process then promotes the release of several pro-inflammatory mediators into the circulation and their binding with hepatocyte cells increases the release of APPs, e.g., nCRP (Kushner and Feldmann, 1978; Zhang et al., 1996; Szalai et al., 1998a; Du Clos and Mold, 2004). Therefore, nCRP is not only an indicator of first-onset stroke but may also be a neurological clinical marker immediately after an IS event (Hertog et al., 2009). A study, carried out in 2014, reported that higher nCRP (>10 mg/L) concentrations were independently associated to a worse initial clinical condition, haematoma growth, neurological worsening and eventually death (Di Napoli et al., 2014). Geng et al. (2017) observed that nCRP levels were significantly and dependently associated to early neurological deterioration in 1,064 patients over 18 years of age, with a BMI between 17.7 and 36.3 kg/m^2. Similar results were confirmed in another review where nCRP levels were associated to an unfavourable long-term clinical outcome, evaluated by the Barthel Index (BI), the Glasgow outcome scale (GOS), the modified Rankin Scale (mRS) and/or the NIH Stroke Scale (NIHSS) (VanGilder et al., 2014).

Several studies have reported that neurological worsening is most likely associated to high nCRP concentrations leading to a larger brain lesion. For example, Jiang et al. (2017b) stated that stroke patients with an nCRP concentration of >6 pg/mL have a larger brain lesion volume three days after a stroke event. You et al. (2018) studied 146 IS patients and confirmed that elevated nCRP (5.4 ± 15.3 mg/L), LDL-C (4.7 ± 1.1 mmol/L) and homocysteine

(26.4 ± 6.3 μmol/L) concentrations were associated to larger white matter lesions (WMLs) and worst clinical cognitive outcomes when these were evaluated by the Montreal Cognitive Assessment scale, the mini-mental state examination and the BI. Kuhlmann et al. (2009) reported that the association between nCRP concentration, larger brain lesions and worse clinical cognitive outcomes probably depends on the capacity nCRP has to bind to ECs through both FcγRIII (CD16) and FcγRII (CD32). When binding takes place, the activation of the MAPKp38 pathways induce TJ protein (occluding and ZO-1) disorganisation, exacerbating BBB destruction (Kuhlmann et al., 2009). This phenomenon was reported to promote an intense leukocyte infiltration and also to induce a stronger, acute, initial pro-inflammatory response, with all the aforementioned negative neurological consequences (Ferrarese et al., 1999; Chiba and Umegaki, 2013; Lu et al., 2018).

In stroke, nCRP may also be used as a middle and long-term neuro-cognitive clinical marker. Together with Erythrocyte Sedimentation Rate, IL-6 and IL-12, nCRP was found to be predictive of post-stroke cognitive impairment (PSCI) (Mijajlović et al., 2017). The relationship between nCRP, stroke and PSCI was also well-documented in a cohort study where a high baseline serum nCRP was associated to a higher risk of developing vascular dementia (Hsu et al., 2017), which is known to be associated to stroke events (Desmond et al., 2002; Iadecola, 2013; O'Brien and Thomas, 2015; Venkat et al., 2015).

1.1.7.5 nCRP and Alzheimer's disease (AD).

Obesity and stroke have often been linked to the risk of developing AD (Businaro et al., 2012; Murali Vijayan and Reddy, 2016; M. Vijayan et al., 2017). AD is a neurological brain disease caused by involving an aggregation of the beta-amyloid protein (beta-amyloid plaques) outside neurons (Carter and Lippa, 2001), together with an aggregation of the tau protein (tau tangles) inside neurons (Murphy and LeVine, 2010). This abnormal protein accumulation interferes with both ordinary transport of neuron nutrients and neuron-to-neuron communication, leading to neural cell death (Niikura et al., 2006) expressed clinically with by its well-known symptoms, i.e., cognitive

problems expressed, thinking/memory, depression, problem-solving and language difficulty (Tarawneh and Holtzman, 2012; Bature et al., 2017).

A study on AD, carried out in 1994, reported that nCRP had been observed in senile plaques in AD patients (Iwamoto et al., 1994). K. Yasojima et al. (2000) that compared mRNA nCRP levels in AD patients and a control group, confirmed the presence of mRNA in the hippocampus of the AD group. Another study reported that elevated nCRP serum concentrations were associated to all causes of dementia when the population studied included subjects over 90 years of age (Kravitz et al., 2009).

1.1.7.6 nCRP and Chronic Kidney Disease (CKD).

Several studies have already reported that the low-chronic grade of inflammation caused by obesity also facilitate the possibility to develop of CKD (Kovesdy et al., 2017). Therefore, it is not surprising that in these NCD elevated nCRP levels have been confirmed. For example, a recent study reported that higher nCRP levels (≥3.0 mg/L) were directly associated with an increased risk of CKD (Gao et al., 2020). Another study on 4,320 participants, average age 54 years, reported elevated nCRP levels (3.2 ± 1.1 mg/L) in CKD patients (Fox et al., 2010). Another study reported that elevated nCRP concentrations (5.40 ± 1.95 mg/L) were inversely proportional to the estimated glomerular filtration rate (eGFR) in 104 CKD patients (Lalramenga et al., 2019).

1.1.7.7 nCRP and atherosclerotic disease.

CKD not only increases nCRP levels but is also a risk factor for atherosclerotic disease (Mathew et al., 2017). Under normal physiological conditions, endothelium cells play a critical role in maintaining the barrier function of the vasculature, by selecting the elements that may cross from blood to tissues (R. A. Vogel et al., 1998; Y. Zhang and Yang, 2016). However, when APR is chronically activated (Rodriguez-Hernandez et al., 2013), this acute-phase reaction may lead to the deterioration of the EC-tight junctions (Pant et al.,

2014). Damage to EC-tight junctions activates both protein kinase C and NF-KB (Willis et al., 2010; Aveleira et al., 2010). This activation increases the secretion of several pro-inflammatory mediators, such as angiotensin II, surface adhesion molecules, monocyte chemoattractant protein-1 (MCP-1/CCL2) and IL-6 (Tousoulis et al., 2006; Xiao et al., 2014; Fatkhullina et al., 2016; Reiss et al., 2017). When MCP-1 is released by ECs (Deshmane et al., 2009), this chemotactic cytokine recruit monocyte cells through the CCR2/CCL2 pathway from the blood stream to the injury site (Sindrilaru and Scharffetter-Kochanek, 2013). This recruitment is facilitated by the presence of E-selectin, P-selectin and L-selectin (Jin et al., 2013), which enhance the rolling and recruitment of leukocyte cells to the injury site (Jin et al., 2013). Once at the site of injury, monocytes differentiate into macrophages (Malissen et al., 2014; Orekhov et al., 2019). The macrophages bind to PAMPs and DAMPs, increasing the secretion of TNF-α, IL-1β and IL-6 (Koh and DiPietro, 2011; Liu et al., 2017a). As aforementioned in paragraph 1.1.3.3, when IL-6 binds to hepatocyte cells (Szalai et al., 1998a; Du Clos and Mold, 2004) it increases nCRP concentration in the circulation (Lowenstein and Matsushita, 2004; Ceron et al., 2005). This increase is followed by nCRP accumulation in the damaged endothelium tissue and if atherosclerotic plaques are present, it contributes to their progression (Paffen and DeMaat, 2006). Autoptic evidence on 302 human cadavers evidenced an nCRP concentration of 1.4 μg/mL in the healthy controls, 2.5 μg/mL in stable plaques, 2.9 μg/mL in plaque erosion and 3.2 μg/mL in acute plaque ruptures (Burke et al., 2002).

nCRP contributes to the evolution of atherosclerotic lesions and plaques in various ways (Paffen et al., 2004). For example, it has been reported that nCRP increases atherosclerosis development by increasing the superoxide and inducible NO (iNOS) concentration and reducing the platelet aggregation inhibitor prostacyclin (PGI2), the expression of endothelial nitric oxide synthase (or NOS3) and the Nitric Oxide (NO) synthesis (Venugopal et al., 2002; Venugopal et al., 2003). When the nCRP reduces the NO concentration (**Fig 1.3**), this has a negative effect on EC survival and activity (Verma et al., 2004). Devaraj et al. (2004) confirmed that when nCRP binds to ECs, this

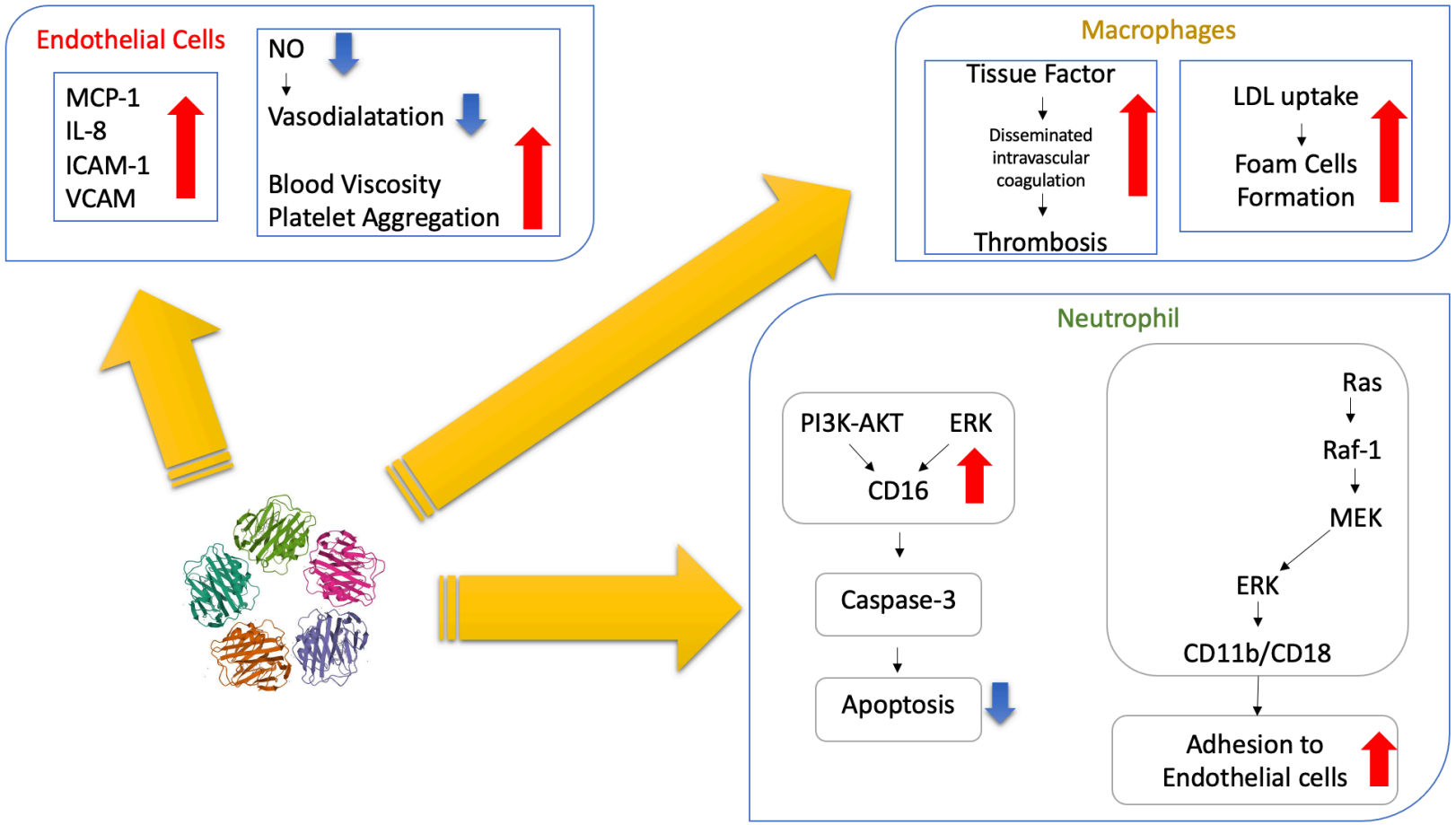


Fig 1.3. nCRP biological activity. As herein reported, nCRP exercises a strong biological activity on several cell types, all of which are involved in cardiovascular disease pathogenesis. For example, nCRP can increase EC and macrophage activation, as well as activation of the complementary cascade. It can also, enhance PAI-1 activity and increase both MCP-1 and IL-8 concentration. Adapted From: Luan Y-Y, Yao Y-M. The Clinical Significance and Potential Role of C-Reactive Protein in Chronic Inflammatory and Neurodegenerative Diseases. Frontiers in immunology. 2018;9:1302

this pentatrix protein activates the NF-KB transcription factor, which promotes IL-8 mRNA and the respective protein translation. Another study corroborated that when nCRP binds to ECs there is an increase in the CD40 and CD40L cell surface expression (Lin et al., 2004), which has previously been proposed as potential markers of inflammation in cardiovascular events (Schonbeck and Libby, 2001).

Two other studies reported that when nCRP binds to ECs there is an increase in the intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1), E- selection and MCP-1 levels (Pasceri et al., 2000b; Pasceri et al., 2001).

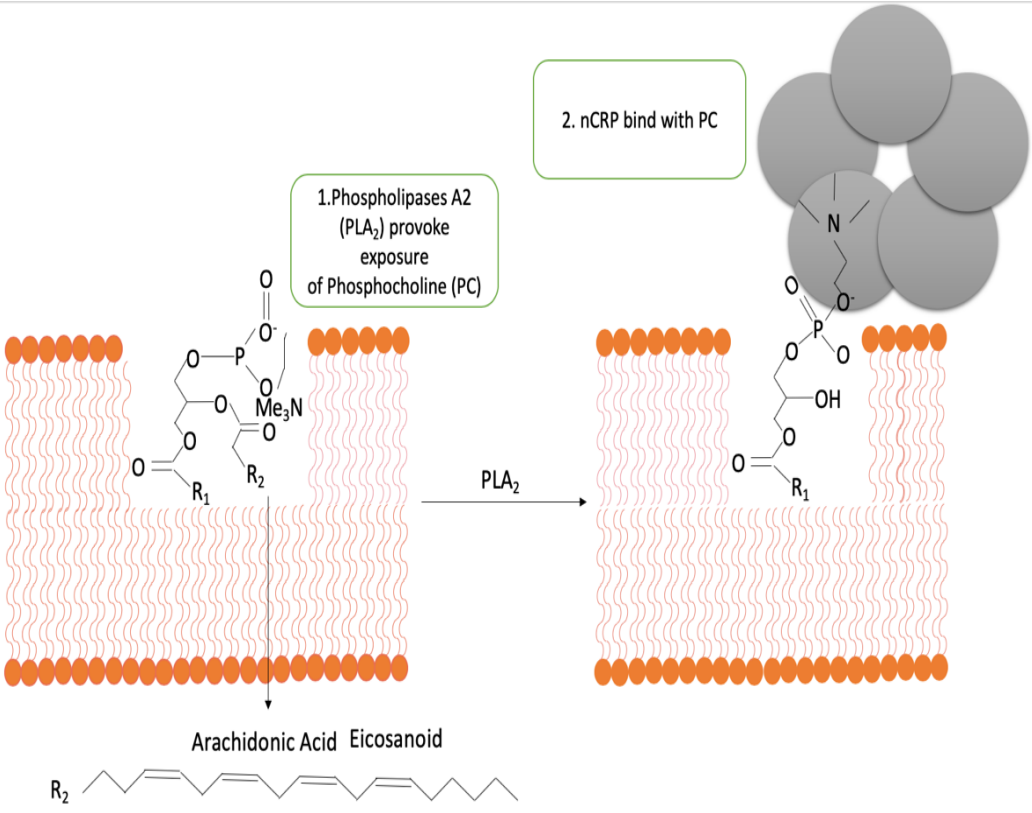
An increase in MCP-1 promotes monocyte binding and rolling to the endothelium through CAMs (Libby, 2002; Hansson, 2005; Hansson and Hermansson, 2011; Jin et al., 2013). Once at the injury site, nCRP facilitates monocyte infiltration into the arterial wall (Torzewski et al., 2000). The nCRP then binds to the monocytes, increasing the production of mRNA and protein product of the macrophage marker CD11b (Yasojima et al., 2001) as well as other several pro-inflammatory cytokines, such as TNF-α, IL-6, IL-1 (Ballou and Lozanski, 1992; Klouche et al., 1998; Paffen et al., 2004).

Along with the recruitment, differentiation and activation of the monocytes, platelets are also stimulated, contributing to the development of an atherogenesis process (Gawaz et al., 2005; Nording et al., 2015). nCRP promotes platelets adhesion to the endothelium (Yaron et al., 2006). However, a study reported that when the nCRP binds to activated platelets it dissociates into the monomeric CRP (mCRP) form (Eisenhardt et al., 2009b).

1.1.7.8 nCRP can be dissociated into a monomeric-CRP (mCRP) form.

Under normal physiological conditions, nCRP maintains its original native pentameric conformational form (Eisenhardt et al., 2009a). However, several studies have also described a monomeric conformational form (mCRP)

originating from the initial nCRP (**Fig 1.4**), which may be obtained both *in vitro* and *in vivo* (Salazar et al., 2014). Two studies reported that nCRP can easily be dissociated by a buffer with 10 mM of EDTA and 8 M/L urea (Potempa et al., 2015; Potempa et al., 1983). Taylor and Van Den Berg (2007) documented how it was possible to dissociate nCRP into mCRP by heating the native protein (nCRP) at 70 °C for one hour. Ji et al. (2007) also reported that the both liposomes and cell membranes dissociate nCRP into mCRP. Later, Eisenhardt et al. (2009b) stated that the nCRP-mCRP dissociation occurs when nCRP binds to the derivates of phosphatidylcholine (PC) exposed on the cell membranes and this dissociation is followed by the accumulation of the mCRP in tissue. Indeed, the presence of mCRP has been confirmed in kidney biopsies of diabetic patients with CKD (Schwedler et al., 2003), in atherosclerosis tissue (Eisenhardt et al., 2009b) and in post- ischemic brain tissue (Slevin et al., 2015).



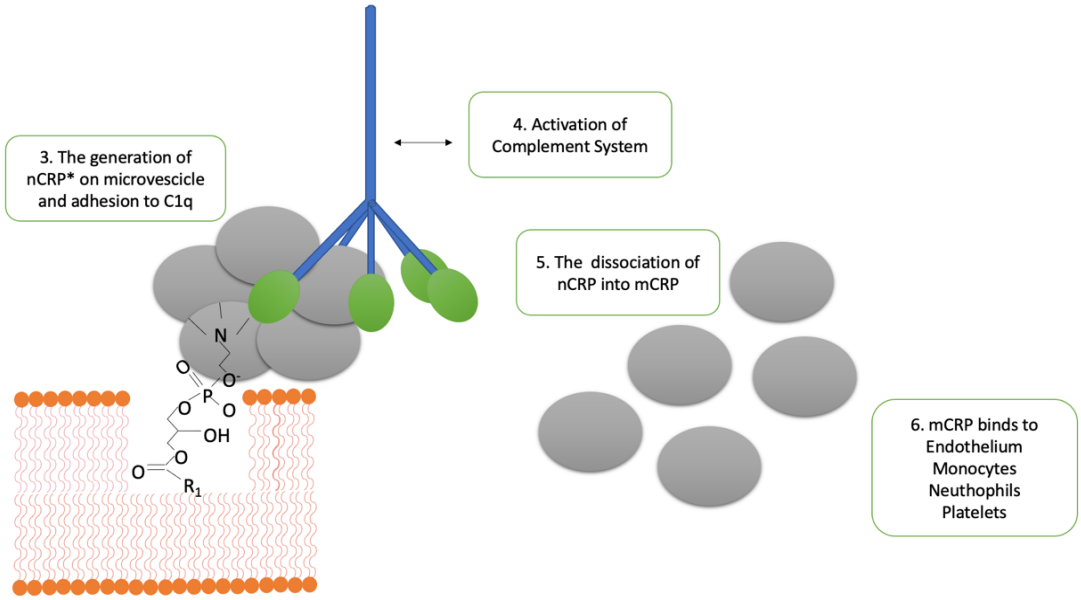


Fig 1.4. nCRP can be dissociated into sub-units or monomeric CRPs (mCRPs). A schematic showing how nCRP is dissociated into mCRP. Phospholipase (PLA2) cause de exposure of phosphatidylcholine (PC). The circulating nCRP binds to the PC exposed on cell membranes. Activated complement system binding with the nCRP dissociates the nCRP into mCRP. This dissociation increases the pro-inflammatory events when the mCRP binds to monocytes, neuthophilis and platelets. Adapted from: Caprio, V., L. Badimon, M. Di Napoli, W.-H. Fang, G. R. Ferris, B. Guo, R. S. Iemma, D. Liu, Y. Zeinolabediny and M. Slevin (2018). "pCRP-mCRP Dissociation Mechanisms as Potential Targets for the Development of Small-Molecule Anti-Inflammatory Chemotherapeutics." Frontiers in immunology 9: 1089.

1.1.7.9 The pro-inflammatory effects of nCRP are triggered by mCRP.

For several years the nCRP was reported to have a strong pro-inflammatory activity. However, this concept has changed substantially after it was reported that the nCRP pro-apoptotic and pro-inflammatory effect may be due to the presence of both sodium azide and/or LPS in the commercial nCRP sample (van den Berg et al., 2004; Albert et al., 2005; Pepys et al., 2005). These publications were followed by other studies which observed that nCRP is characterised by both pro and anti-inflammatory activities while mCRP mainly featured pro-inflammatory proprieties on different cell types, such as neutrophils, platelets and ECs. Khreiss et al. (2002) reported that, contrary to nCRP, the binding of mCRP to FcγRIII (CD16) activates the MAPK/ERK and PI3-Kinase/Akt signalling pathways, inhibiting the apoptotic process and decreasing Deoxyribose Nucleic Acid (DNA) fragmentation in neutrophils. Later, Tarek et al. (2004) showed that, differently to nCRP, mCRP is capable

of increasing neutrophil adhesion to human coronary ECs (HCAECs) when it binds to the FcγRIII (CD16) receptors. This process activates the MAPK-MAPKp38 intrinsic pathway, which triggers the synthesis and release of pro-inflammatory mediators, such as IL-8, MCP-1, E-selectin, ICAM-1 and VCAM-1 (Khreiss et al., 2004a). Furthermore, Tarek et al. (2006) reported that mCRP increases IL-8 secretion through the activation of both NF-KB and activator protein-1 (AP-1) in human neutrophil cells.

In platelets, Molins et al. (2008) stated that, differently to nCRP, mCRP is capable of: *(I)* increasing the activation marker P-selectin; *(II)* increasing their deposition; *(III)* and also increasing thrombus frequency.

mCRP has been shown to have pro-angiogenic effects in ECs, through the activation of both extracellular signal regulated kinase (ERK) (Turu et al., 2008) and the PI3K/Akt survival intrinsic pathway (Boras et al., 2014). Peña et al. (2017) reported that human microvascular ECs (HMECs) mCRP can: *(I)* encourage angiogenesis *in vivo*; *(II)* promote endothelia tube formation; *(III)* promote mEC motility; *(IV)* and increase MCP-1 synthesis and release through the activation of the AKT intrinsic pathway and the ETS1 transcription factor. In another study by Molins et al. (2017) it was reported that mCRP *(I)* affects the ARPE-19 cell viability, *(II)* facilitates paracellular permeability, *(III)* decreases transepithelial electrical resistance, *(IV)* and perturbs the expression of both ZO-1 and occluding in retinal pigment epithelium cells.

1.1.7.10 Mitogen-activated protein kinase (MAPK) and Nuclear factor K chain transcription in B cells (NF-KB).

As previously reported in paragraph 1.1.7.9, several studies reported that the mCRP pro-inflammatory activity is triggered when MAPKs or NF-KB are activated (Khreiss et al., 2002; Tarek et al., 2002; Tarek et al., 2004; Tarek et al., 2006; Turu et al., 2008).

MAPKs are proteins able to control essential cellular procedures, such as stress responses, apoptosis and immune defence (Dong et al., 2002; Wada

and Penninger, 2004; Ai et al., 2016). Literature has reported that MAPKs are one of the most important cascade signals in all eukaryotic animals, fungi and plants (Bardwell, 2006). This evolutionary conserved, intracellular signal transduction pathway has the pivotal role of controlling numerous fundamental cellular processes, such as stress response and inflammation (Kaminska, 2005; Cargnello and Roux, 2011).

As shown in (**Fig 1.5**), when Cytokines, PAMPs, DAMPs, TGF-β1, hormones or growth factors stimulate MAP kinase kinase kinases (MAPKKKs) (Liu et al., 2007; Kyriakis and Avruch, 2012; Johnson and Lapadat, 2002). In turn, MAPKKKs activate MAP kinase kinase (MAPKK) (Morrison, 2012). MAPKK then phosphorylates and activates the last MAP kinases (MAPKs) (Zhou et al., 2010). MAPKs can be dived into three different MAPK pathways, known as the extracellular signal regulated kinase (ERK), c-JUN N-terminal kinase (JNK) and the Mitogen-activated protein kinase p38 (MAPKp38) (Lu and Xu, 2006; Zarubin and Han, 2005).

The first MAPK to have been elucidated was the extracellular signal regulated kinase (ERK1/2) (Seger and Krebs, 1995). According to literature, this intrinsic pathway is usually (but not only) triggered by the activation of small G proteins (e.g., Ras), Ca^{2+}activation and/or PKC-mediated activation (Lawrence et al., 2008). When this occurs, the signal is propagated by the activation of the protein kinases Raf-1, B-Raf and A-Raf (Rafs), which are all serine/threonine-specific protein kinases belonging to the MAPKKKs level of this intrinsic pathway (Kyriakis et al., 1993; Wellbrock et al., 2004). After this initial activation, the signalling is diffused through the activation of MAPKKs (MEKs) (Ahn et al., 1991; Gómez and Cohen, 1991; Kyriakis et al., 1992), which are two proteins with molecular weight of 45 kDa and 46 kDa (MEK1 and MEK2 respectively) (Wortzel and Seger, 2011). When MEKs, i.e., MEK1 and MEK2 (Caunt et al., 2015) are activated after phosphorylation of Ser298 and Ser386 (Nadeau et al., 2009), in turn, these protein kinases phosphorylate the Tyr and Thr of ERKs (Seger et al., 1992; Seger et al., 1995). ERKs are two evolutionary conserved MAPKs (ERK1 and ERK 2) synthesised by two different genes (Mapk3 and Mapk1 respectively) (Upadhya et al., 2013) which often has

similar regulation and function (Buscà et al., 2016). After being stimulated, ERK1 and ERK2 phosphorylate other substrates present in the cytoplasm (Lenormand et al., 1993). Literature has reported that ERK1/2 are responsible for the induction and regulation a massive quantity of substrate (Yoon and Seger, 2006; Daub et al., 2008), most of them are localized in the Golgi apparatus, cytoplasm, mitochondria, endosomes/lysosomes and various other membranes (Plotnikov et al., 2011). This substrate may enter into the nucleus and stimulate gene expression in reaction to the previous extracellular stimuli regulating the cell proliferation, differentiation, apoptosis and inflammation response (Deming et al., 2008; Papa et al., 2019; Guo et al., 2020). When the activation of ERKs is associated with inflammation, this intrinsic pathway increases NF-KB and activator protein-1 (AP-1) activity (Mandrekar and Szabo, 2009).

The c-JUN N-terminal kinase (JNK) cascade is also referred to as stress-activated protein kinases (SAPKs) (Dunand-Sauthier et al., 2005). This intrinsic pathway was originally recognized as a regulator of the transcription factor c-Jun (R. J. Davis, 1994; Xia et al., 1995). Several stimuli can lead to the activation of JNK, such as bacteria, growth factors, heat shock inflammatory cytokines and UV radiation (Hamdi et al., 2005; Bogoyevitch and Kobe, 2006; Seki et al., 2012). When this stimulus binds to cells, they activate several MAPKKKs, such as apoptosis signal-regulating kinase 1 (ASK-1), mixed lineage kinase (MLK) 2/3, and transforming growth factor β-activated kinase (TAK) 1 (Davis, 2000). Upon activation, MAPKKKs transmit the signals by phosphorylating Thr and Ser residue, which, in turn, leads to MAPKK (MKK4 and/or MKK7) activation (Wang et al., 2007). When MKK7 (also known as MEK7) is activated, this protein kinase phosphorylates the JNK intrinsic pathway (Park et al., 2019). When MKK4 (also known as MEK4) is phosphorylated, this protein kinase is not only able to phosphorylate and activate all three JNK isoforms (JNK 1/2/3) but can also stimulate the MAPKp38 signalling pathway (Brancho et al., 2003), another stress-activated MAPK cascade (Whitmarsh and Davis, 2007). After phosphorylation, JNK can stimulate several transcription factors, such as AP1, activating transcription

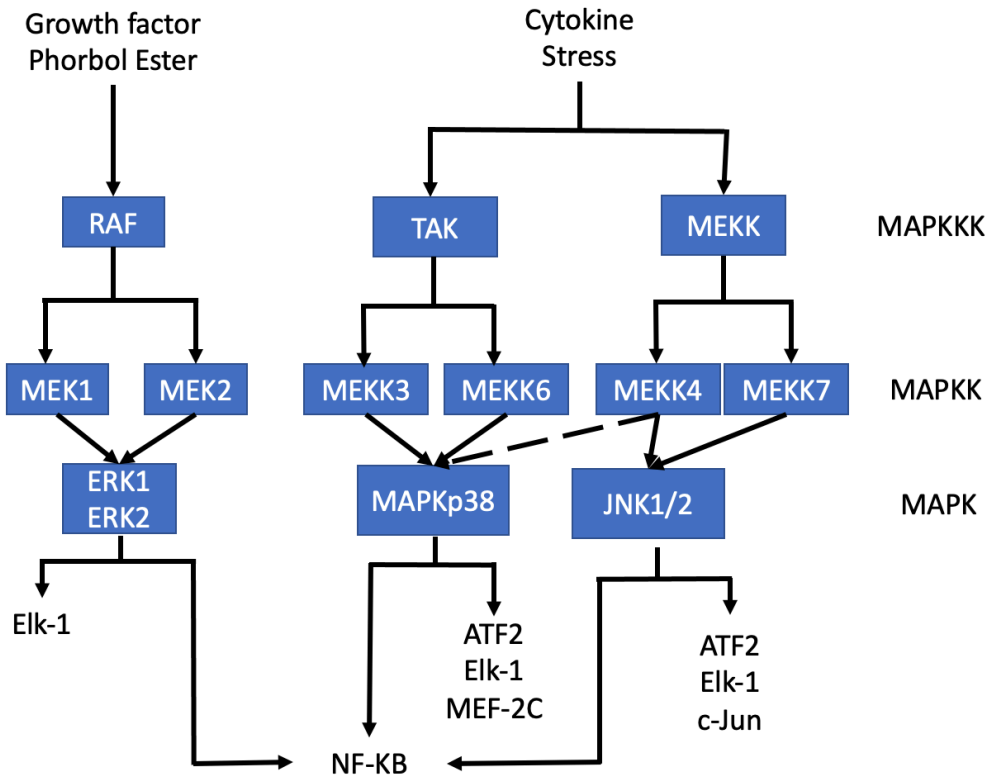


Fig 1.5. The Mitogen-Activated Protein Kinase (MAPK) Pathway. A pathway showing extra-cellular stimuli activate MAPKKKs, this serine/threonine-specific protein kinase activates MAPKK. MAPKKs in turn activate, the MAPKs (ERK1/2, JNK1/2 and MAPKp38). The phosphorylated MAPK signalling pathways induces the activation and the translocation of the NF-KB transcription factors. Adapted from: Dong, C., R. J. Davis and R. A. Flavell (2002). "MAP KINASES IN THE IMMUNE RESPONSE." Annual review of immunology 20(1): 55-72.

factors (ATF) and the ETS Like-1 protein (Elk1) (Chadee and Kyriakis, 2010) and NF-KB (Maeda, 2010).

MAPKp38 is another MAPK divided into MAPKp38α (MAPK14), MAPKp38β (MAPK11), MAPKp38γ (MAPK12/ER- K6) and MAPKp38δ (MAPK13/SAPK4) pathways (Zarubin and Han, 2005; Lu and Xu, 2006). MAPKp38 are activated by environmental and cellular stress conditions induced by pathogens (virus and bacteria), growth factors, heat shock and/or cytokines (Obata et al., 2000). Taking in consideration that this MAPK is activated by the same stimuli present in MAPK/JNK, it is not surprising that this intrinsic pathway is stimulated by the same MAPKKKs, i.e., MLK 2/3, ASK1 and TAK1 (Harada et al., 2006; Handley et al., 2007; Sakurai, 2012). Similar to other MAPKs, when MAPKKKs i.e MLK 2/3, ASK 1 and TAK1 are stimulated they induce phosphorylation and the activation of MAPKKs i.e., MEKK3, MEKK4 and MEKK6 (Tanaka et al., 2002; Craig et al., 2008; Park et al., 2019). Literature reports that MEKK3 is a protein kinase, belonging to the MEKK/STE11 family (Blank et al., 1996; Su et al., 2001) and is capable of stimulating the MAPKp38α, MAPKp38β and MAPKp38δ signalling pathways (Y. Jiang et al., 1997), while MKK6 is a MAPKK that stimulates all MAPKp38 isoforms i.e., MAPKp38α, MAPKp38β, MAPKp38γ, and MAPKp38δ pathways (Eyers et al., 2005). Once MAPKp38 is stimulated, this protein kinase activates several substrates or transcription factors. Amongst these, literature reported that the MAP kinase-activated protein kinase 2 (MAPKAPK2) (Mahtani et al., 2001) and MAP kinase-activated protein kinase 3 (MAPKAPK3) (McLaughlin et al., 1996) are specifically activated by MAPKp38. When one of these two substrates is phosphorylated, these MAPKAPKs induce the cAMP response element-binding protein (CREB) (Maizels et al., 2001) and/or small heat shock protein 27 (HSP27) activation (Vidyasagar et al., 2012). Furthermore, Moens et al. (2013) reported that MAPKAPKs also stimulate the transcription factor ATF-1, the MAPK-interacting kinases (MNK1 and 2), the mitogen- and stress-activated kinase (MSK) 1 and 2, and p38-regulated/activated kinase (PRAK). Moreover, there are several transcription factors MAPKp38 stimulates, including myocyte enhancer factor 2 (MEF2) (Han and Molkentin, 2000), the

ATF-1 and ATF-2 (LaJevic et al., 2011), the E26 transformation-specific sequence-1 (ETS-1) (Liu et al., 2017b) and NF-KB (Xiao et al., 2020).

The phosphorylation of the MAPK intrinsic pathway is a fundamental step for the stimulation of numerous protein kinases and TFs (Liu et al., 2007; Wei et al., 2020). Amongst the different TFs, several studies have reported that MAPKs are able to stimulate NF-KB (Tak and Firestein, 2001; Sun et al., 2013b; Martinez et al., 2016). NF-KBs are transcription factors (**Fig 1.6**) protein

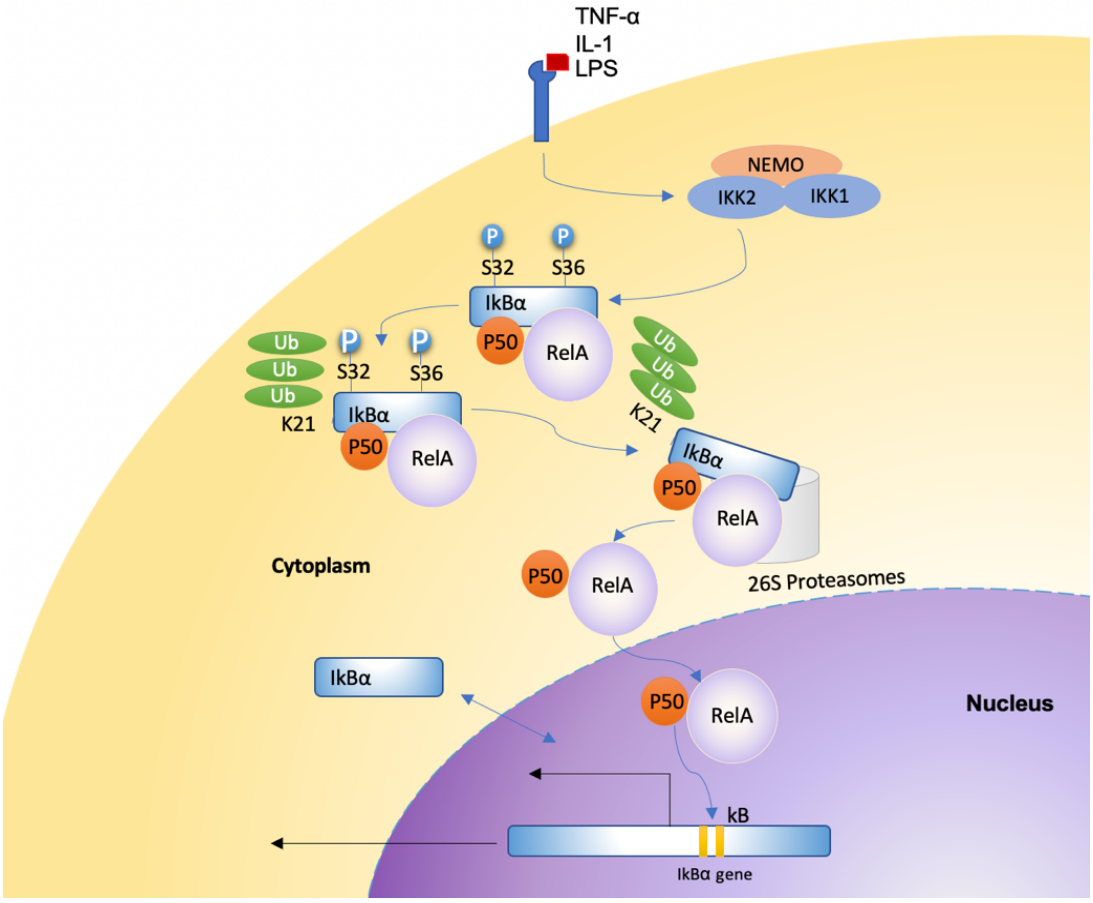


Fig 1.6. The transcription factor Nuclear factor K chain transcription in B cells (NF-KB). Cells are activated by TNF-α, IL-1 and/or LPS. This induces IκBα phosphorylation (P) mediated by the signal (NEMO-IKK1-IKK2 macromolecular complex). IKK2 promotes IκBα phosphorylation, adding ubiquitin molecules (ubiquitylation) and disintegrating the cytoplasmic inhibitor (IκBα) by the 26S proteasome. Degradation of the IκBα cytoplasmic inhibitor allows the NF-KB to translocate into the nucleus. Adapted from: Chen L-F, Greene WC. Shaping the nuclear action of NF-KB. Nature Reviews Molecular Cell Biology. 2004;5(5):392-401.

complexes identified in seven different proteins, i.e., p105, p100, p50, p52, RELA/p65, c-REL RELB originated from five different NF-KB/REL genes, i.e., NFKB1, NFKB2, RELA, c-REL, and RELB (Chen and Greene, 2004). Under physiological conditions, (**Fig 1.6**) the NF-KB complex is located in the

cytoplasm attached to its inhibitory IκBα kinase cytoplasmic inhibitor (Hayden et al., 2006b). The IκBα kinase cytoplasmic inhibitor allows the NF-KB to enter into the nucleus and to regulate the gene expression only when an external stimulus degrades the IkB protein, by triggering a sequence of specific steps (Oeckinghaus and Ghosh, 2009). Once in the nucleus, NF-KB up-regulates the gene expression of several pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6 (Hayden et al., 2006b; Hayden and Ghosh, 2008; Lawrence, 2009; Tanaka et al., 2014; Pires et al., 2018). Several studies demonstrated that both MAPK and NF-KB pathways are triggered when monocytes and macrophages carry out their fundamental role during the inflammatory phase, due to their capacity to secrete several pro-inflammatory cytokines (Liu et al., 2003; Liu et al., 2017a).

1.2 Monocytes and Macrophages.

1.2.1 Monocytes.

One of the first studies on monocytes evidenced that mature monocytes differentiated into tissue macrophages when they migrated from the circulation to the organs (van Furth and Cohn, 1968). Much has been done since that first study to better clarify the origin and differentiation of monocytes (**Fig 1.7**). Nowadays, it is known that a human monocyte *(I)* originates from a haematopoietic stem cell (HSC), *(II)* becomes a common myeloid progenitor (CMP), *(III)* further develops into a granulocyte-macrophage progenitor (GMP), *(IV)* then progress to a macrophage and DC progenitor (MDP) and *(V)* then develops into a monocyte (Chow et al., 2011).

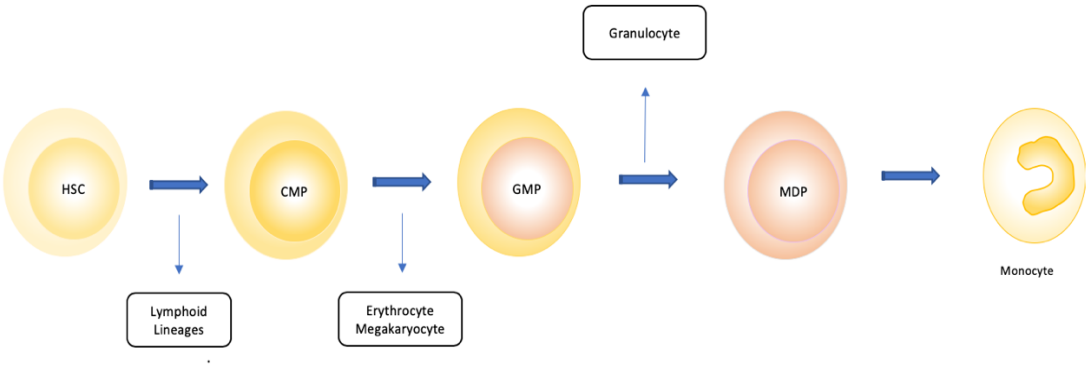


Fig. 1.7. The origin of monocytes. When a haematopoietic stem cell (HSC) becomes a common myeloid progenitor (CMP), it then develops into a GMP, matures into a macrophage and DC progenitor (MDP) and, in the end, it further evolves to become a monocyte. The monocytes subsequently differentiate when they are recruited from the peripheral bloodstream. Adapted from: Chow A, Merad M, Brown BD. Studying the mononuclear phagocyte system in the molecular age. Nature Reviews Immunology. 2011;11(11):788-98.

1.2.1.1 Monocyte Subsets.

The Nomenclature Committee of the International Union of Immunological Societies subdivided human monocytes into three major populations (Ziegler-Heitbrock et al., 2010), "$CD14^{++}$ $CD16^{-}$ monocytes" (also known as classical monocytes), "$CD14^{+}$ $CD16^{+}$ monocytes" (also known as intermediate monocytes) and $CD14^{+}$ $CD16^{++}$ monocytes (also known as non-classical monocytes) (Sampath et al., 2018; Kapellos et al., 2019). This classification was based on distinct phenotypical and anatomical characteristics. For

example Boyette et al. (2017) reported that: *(I)* the classical and intermediate monocytes mainly expressed the CD11b surface marker, whilst the non-classical monocytes expressed the CD11c and CX3CR1 surface markers; *(II)* classical monocyte subtypes were described as being bigger than the non-classical ones; *(III)* only classical and intermediate subtypes can express pro-inflammatory cytokines, such us IL-1β, IL-6 and TNF-α; *(IV)* the classical monocyte subtype was found to be the only subset that could differentiate itself into monocyte-derived dendritic cells (DCs), whilst all the other three groups could only differentiate themselves into monocyte-derived macrophages. Ogle et al. (2016) reported that the classical monocyte subtype is a subgroup that *(I)* arises from both bone marrow and spleen, *(II)* expresses the CC-chemokine receptor (CCR2), *(III)* are recruited from the blood to the injured site when the CC-chemokine ligand 2 (CCL2 also know MCP-1) in the blood binds to classical monocytes via CCR2 receptors. Furthermore, it was reported that the classical monocyte subtype is recruited during CCR2 activation in the presence of the CCL7 protein (also known as MCP-3), which was highly expressed during infections and inflammation conditions, induced by bacterial, protozoal, fungal, viral infections and damage tissue (Shi and Pamer, 2011). Another paper stated that once recruited and activated, this cell subtype eliminates the bacteria by boosting the concentration of both inducible nitric oxide synthase (iNOS) and NO, which induces DNA damage and affects their metabolism (Xiong and Pamer, 2014). Ogle et al. (2016) reported that once the pro-inflammatory cell phenotypes conclude their activity they leave their place to the non-classic monocytes, which are able to assist and repair the damaged tissue during the last phase of wound healing process (WHP) (Ogle et al., 2016).

1.2.1.2 Human Monocyte Subsets in Major Chronic Inflammatory Diseases.

Over the last few years, several studies have reported that life-style habits a have a significant effect on the number of circulating monocytes (Kapellos et al., 2019). Indeed, numerous studies have evidenced that there is a notable

increase of circulating monocytes in several NCDs (Ozanska et al., 2020). A high monocyte count (MC) was observed in several chronic inflammatory conditions, e.g., obesity, CKD, arteriosclerosis and stroke (Rogacev et al., 2014; Chistiakov et al., 2018), where the presence both nCRP/mCRP was also confirmed (Schwedler et al., 2003; Eisenhardt et al., 2009b; Thiele et al., 2014a; Thiele et al., 2015; Slevin et al., 2017).

For example, as aforementioned in chapter 1 paragraph 1.1.7.1. in obesity, the AT cells release pro-inflammatory mediators, such as PAI-1, HMGB-1 and MCP-1 (Makki et al., 2013; Guzmán-Ruiz et al., 2014). Indeed, MCP-1 is a constantly present chemoattractant protein in the systemic circulation of the obese population (Panee, 2012). An increase in MCP-1 is often associated with an increase in MC count (Yoshimura et al., 2015). A study carried out in 2002 on 300 obese subjects reported a higher average MC (13–18%) in the obese group than in the healthy control group (54 subjects) (Kullo et al., 2002). Schipper et al. (2012) reported that 60 obese children, aged 6 to 16, with a BMI of >30 kg/m^2, had a *(I)* low insulin sensitivity and low HDL-cholesterol levels; *(II)* but an elevated systolic blood pressure; *(III)* an elevated presence of several pro-inflammatory mediators; *(IV)* a high nCRP concentration of >1.70 mg/L; *(VI)* and an increase in their MC, especially the classical pro-inflammatory ($CD14^{++}CD16^{-}$) monocyte subtype. Pecht et al. (2014) reported that the MC is directly associated with obesity and its complications. Devevre et al. (2015) evidenced that their obese population had a higher MC than normal-weight nondiabetic volunteers. The same study also reported that the monocytes collected from the obese population released more pro-inflammatory mediators when stimulated by LPS or viral ssRNA, than healthy subjects (Devevre et al., 2015). Similar results were also reported in a study by Leite et al. (2017) who evidenced that obese patients, aged 20 to 63 years with a BMI of >30 kg/m^2 had higher: *(I)* leptin plasma levels (1.17 ng/ml); *(II)* systolic blood pressure (129 ± 16 mm Hg, F) and diastolic blood pressure (89 ± 9 mm Hg, F); *(III)* nCRP concentration (3.020 mg/L); *(IV)* and the non-classical monocyte subtypes ($CD14^{+}$ $CD16^{++}$) also had a higher inflammatory pattern than that of the healthy non-obese control group. Choi et al. (2017) studied 439 men and 561 women over 65-years of age and observed a high

BMI and white blood cell count, serum nCRP levels of 0.25 mg/dL and a high MC, all of which were associated with a high risk of death. Wouters et al. (2017) used flow cytometry to compare 29 obese male patients, aged 45.0 to 54.0 years, with a BMI of >30 kg/m2, to a healthy control group. They confirmed that the percentage of both classical and nonclassical monocytes was higher in the obese group than in the healthy controls (Wouters et al., 2017). In a recent study, Friedrich et al. (2019) reported the presence of the three monocyte phenotypes in their obese study group, which included 60 obese patients with a BMI of ≥ 30 kg/m^2 aged 45.0 to 47. They evidenced an elevated HbA1c-IFCC, nCRP (10.4 ± 0.9 mg/mL) and leukocyte and monocyte concentration. Furthermore, Friedrich et al. (2019) reported that there was a statistically significant association between the absolute number of $CD14^{++}CD16^{-}$ pro-inflammatory classical monocytes and fat mass.

The increase in monocyte counts due observed in obesity also explains why a rise in these white blood cells could be considered a prognostic marker of several obesity-related diseases, such as CKD, arteriosclerosis and stroke (Gkrania-Klotsas et al., 2010; Zhang et al., 2017b; Kovesdy et al., 2017; Authier et al., 2020). For example, Wallquist et al. (2013) studied 12 patients with CKD and observed an increase in $CD16^{+}$ monocytes in the circulation. Similar results were also reported by Bowe et al. (2017) who confirmed that elevated MCs had a statistically significant correlation with CKD. Rogacev et al. (2014) reported on 438 CKD patients with elevated $CD14^{++}CD16^{+}$ monocyte counts, which were predictive of cardiovascular events. It is known that patients with CKD run a higher risk of developing some cardiovascular diseases, such as atherosclerosis (Kon et al., 2011), another chronic disease where the MC count is alternated. Indeed, it has also been suggested that MC is a useful predictor of subclinical carotid arteriosclerosis in both genders with an average age of 53.3 ± 13 years (Chapman et al., 2004). Johnsen et al. (2005) reported on 2,610 healthy subjects aged 25 to 82 years without arteriosclerotic plaques, their 7-year follow-up evidenced that age, gender, total cholesterol, especially MC, were all predictors of future arteriosclerotic plaque formation. Later, Rogacev et al. (2010) reported on the relationship the three monocyte phenotypes have with obesity and arteriosclerosis. They

studied total of 569 subjects and observed that their BMI had a positive association with $CD16^+$ (intermediate and non-classical) monocytes but not with $CD16^-$ monocytes. Furthermore, they also confirmed that in those in the obese group with a BMI of ≥ 35 kg/m^2, the $CD16^+$ monocytes were two-fold that of the healthy control group (Rogacev et al., 2010). Similar results were also confirmed by Poitou et al. (2011) who reported that 144 obese patients with a BMI range of 25.3 to 68.6 kg/m^2 and elevated nCRP (> 4.4 mg/mL) also had an elevated concentration of intermediate and non-classical monocyte phenotype, which, in turn, were also associated with a higher risk of developing arteriosclerosis. Berg et al. (2012) reported there was a linear association between an elevated classic monocyte ($CD14^{++}CD16^-$) concentration and a higher incidence of coronary events, IS and CVD. The same study also evidenced that this association was particularly strong even when these diseases were not associated with gender, age, smoking, HDL cholesterol, diabetes and/or hypertension (Berg et al., 2012). Yang et al. (2014a) reported that there was also an increase in MC in other pathologies, such as coronary arterial disease (CAD), left ventricular dysfunction and left ventricular aneurysm. More recently, Kim et al. (2017) reported that in people with an BMI >24.5 kg/m^2, there was a linear association with total WBC count, age, BMI and nCRP levels. Furthermore, the same study confirmed that the WBC count was specifically associated to a higher risk of coronary artery atherosclerosis and cardiovascular disease, due to an increase in MC (Kim et al., 2017). Therefore, it is not surprising that several studies have also reported an increase in MC in some cardiovascular disease, such as IS. Indeed, a statistically significant increase in MC was observed by Urra et al. (2009) in 45 stroke patients evaluated 180 minutes after the of stroke onset, compared with the healthy controls. Kaito et al. (2013) reported that IS increases MC (especially the classical monocyte subset $CD14^{++}CD16^{-}$) in the circulation. Moreover, an elevated MC has been also associated with an elevated nCRP concentration in IS (Chiba and Umegaki, 2013; Liberale et al., 2017). Similar results were also reported by Dong et al. (2020) who reported that an elevate MC after an IS event is associated to a poor outcome.

An MC increase has also been associated to an increase in recruitment infiltration and monocyte accumulation from the circulation to the tissue affected by disease itself respectively, in all these medical conditions (Woollard and Geissmann, 2010; Chiba and Umegaki, 2013; Malissen et al., 2014; Naicker et al., 2018). This accumulation is immediately followed by a differentiation from monocytes to monocyte-derived macrophages (BMdM) (Bobryshev et al., 2016; Guiteras et al., 2016; Zhang et al., 2019).

1.2.2 Macrophages.

Macrophages can be classified into two different groups: Tissue resident macrophages (TRM) and blood monocyte-derived macrophages (BMdM). For the sake of simplification, the term *macrophage* will be used to indicate blood monocyte-derived-macrophages in this paragraph.

1.2.2.1 Monocytes differentiation into macrophages.

Monocytes are recruited around the injury site when TRM, fibroblasts and endothelial cells (ECs) release CCL2 and CX3CL1 and the CCR2/CCL2 and CX3CR1/CX3CL1 pathways are activated (Deshmane et al., 2009; Sindrilaru and Scharffetter-Kochanek, 2013). This recruitment is also facilitated by the presence of cell adhesion molecules (CAMs) expressed on ECs which enable the monocytes to bind and roll on the endothelium, which helps them reach the injured site (Chi and Melendez, 2007; Hansson and Hermansson, 2011; Jin et al., 2013). Once at the injury site, monocytes extravasate into the surrounding area and express specific surface makers, such as F4/80, CD11b, CD18, CD68, colony stimulating factor 1 receptor (CSF1R), pathogen recognition receptors (PRRs) and FC receptors (Geissmann et al., 2010; Murray and Wynn, 2011; Malissen et al., 2014), they then differentiate into a naïve M0 resting macrophage phenotype (Orekhov et al., 2019). This macrophage accumulation has been confirmed in several tissues specifically affected by several NCDs where the presence of both nCRP and mCRP has also been observed. Indeed, it has been reported that the presence of macrophages in the kidney is a common finding in chronic kidney disease

(CKD) and the amount of macrophage accumulation is associated with the severity of renal injury (Wang and Harris, 2011). Macrophage accumulation in CKD was also confirmed in a study which reported that these immune system cells also play a pivotal role in CKD progression (Ohara et al., 2019). This fundamental role was also reconfirmed in a recent review by Engel and Chade (2019) where the authors also add that the manipulation of these immune system cells may provide a potential therapy against CKD.

CKD, is a chronic disease often associated to atherosclerosis, another chronic medical condition where the local accumulation of monocyte-derivate macrophages in arteriosclerotic inflamed tissue has been also confirmed (Kon et al., 2011; Olechnowicz-Tietz et al., 2013; Balla et al., 2013). Indeed, Zeller and Srivastava (2014) reported that, after monocyte recruitment, these cells differentiate into monocyte-derived macrophages in the sub-endothelial space artery. Flynn et al. (2019) stated that the majority of macrophages present in arteriosclerotic plaques originate from the infiltration of circulating monocytes into arteriosclerotic plaque tissue (Flynn et al., 2019). Moreover, Barrett (2020) stated that the accumulation of monocyte-derived macrophages is so elevated the macrophage count increases by up to 20-fold in the aorta during the atherogenesis process. This exponential increase is probably associated to the pivotal role monocyte-derived macrophages play during atherosclerosis progression (Flynn et al., 2019).

It is known that atherosclerosis increases the risk of IS events (Banerjee and Chimowitz, 2017) and, not surprisingly, an accumulation of monocyte-derived macrophages has been also confirmed in this condition (Chiba and Umegaki, 2013). Indeed, Kim and Cho (2016) observed an accumulation of both monocytes and monocyte-derived macrophages after acute IS injury. Khan et al. (2016) reported that a provoked IS event, in an adult male mouse (10-12 weeks), through a distal middle cerebral artery led to an accumulation of monocyte-derived macrophages in its ischemic brain tissue (IBT). Similar results were also confirmed by Pedragosa et al. (2018) in IBT. They reported that an IS event provoked in an adult male mouse, through a right middle cerebral artery occlusion, confirmed an increase in monocyte-derived

macrophage accumulation in IBT (Pedragosa et al., 2018). A recent review also evidenced an accumulation of monocyte-derived macrophages in IBT (Jian et al., 2019).

1.2.2.2 Two macrophages subtypes.

Macrophages polarize from the M0 phenotype into pro or anti-inflammatory (M1 or M2 respectively) phenotypes depending on the stimuli they receive from in the injury site (Chistiakov et al., 2015; Ley, 2017). Pro-inflammatory cytokines, LPS, DAMPs and PAMPs enable the resting M0 phenotype to evolve into the M1 classically activated phenotype, which secretes pro-inflammatory cytokines (IL-1, IL-6, IL-12, TNF-α) and iNOS (Koh and DiPietro, 2011; Liu et al., 2017a). An *in vivo* investigation by Mirza and Koh (2011) reported an increase in the pro-inflammatory macrophage M1 (which expressed a high concentration of IL-1β, MMP-9 and NOS) on day 5, in an 8-millimetre excisional wound. However, the same authors observed that there was a substantial decrease in the pro-inflammatory mediators after a rise in anti-inflammatory markers (CD206 and CD36), insulin growth factor 1 (IGF-1), transforming growth factor-β (TGF-β) and vascular endothelial growth factors (VEGF) on day 10 (Mirza and Koh, 2011). The polarisation from M1 macrophages to M2 macrophages appears to be promoted by basophils when these cells release interleukin-4 (IL-4) and interleukin-13 (IL-13) near the injury site (Egawa et al., 2013; Kang and Biswas, 2013; Van Dyken and Locksley, 2013).

These two interleukins activate the arginase pathway (**Fig 1.8**) and boost the arginine metabolism, triggering L-ornithine, polyamine and proline activity (Mills et al., 2000; Mills, 2001; Gordon, 2003). The polyamine and proline activity increases the secretion of IL-10, arginase-1, collagen and the fibroblast stimulating factor typically release by the M2 macrophages (Ogle et al., 2016; Ley, 2017). However, recent studies have demonstrated that both nCRP and mCRP affect macrophages by facilitating or interfering with this polarization process (Moore et al., 2001; Trial et al., 2016; Pilling et al., 2017).

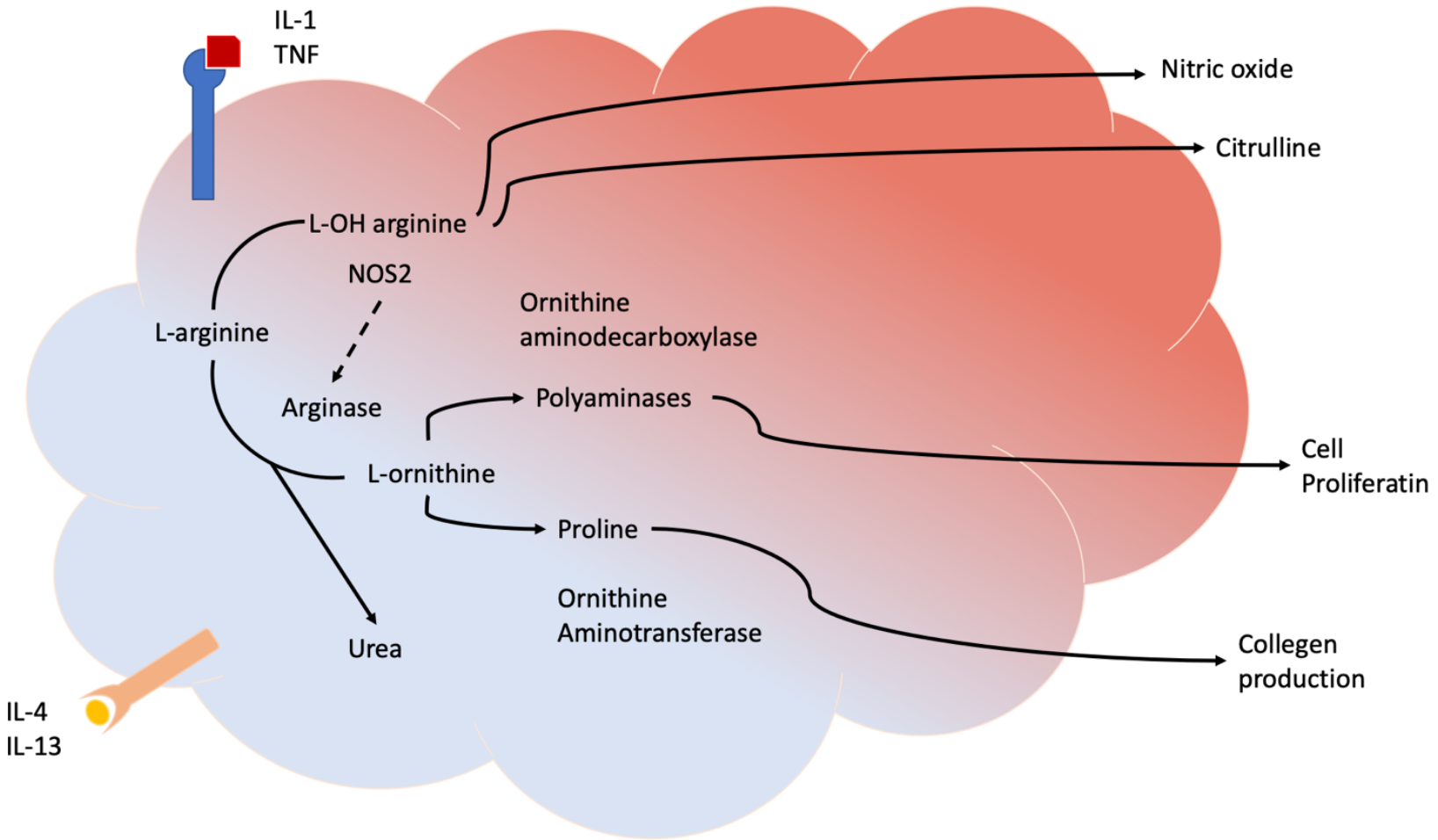


Fig 1.8. Macrophages polarisation: from M1 to M2. When IL-4 and IL-13 boost the arginine metabolism to L-ornithine, the polyminase and proline activity is intensified. The activation of these two pathways allow the cell to polarize into an anti-inflammatory phenotype. These cells then release collagen and facilitate cell proliferation. Adapted from: Gordon, S. (2003). "Alternative activation of macrophages." Nature Reviews Immunology 3(1): 23-35.

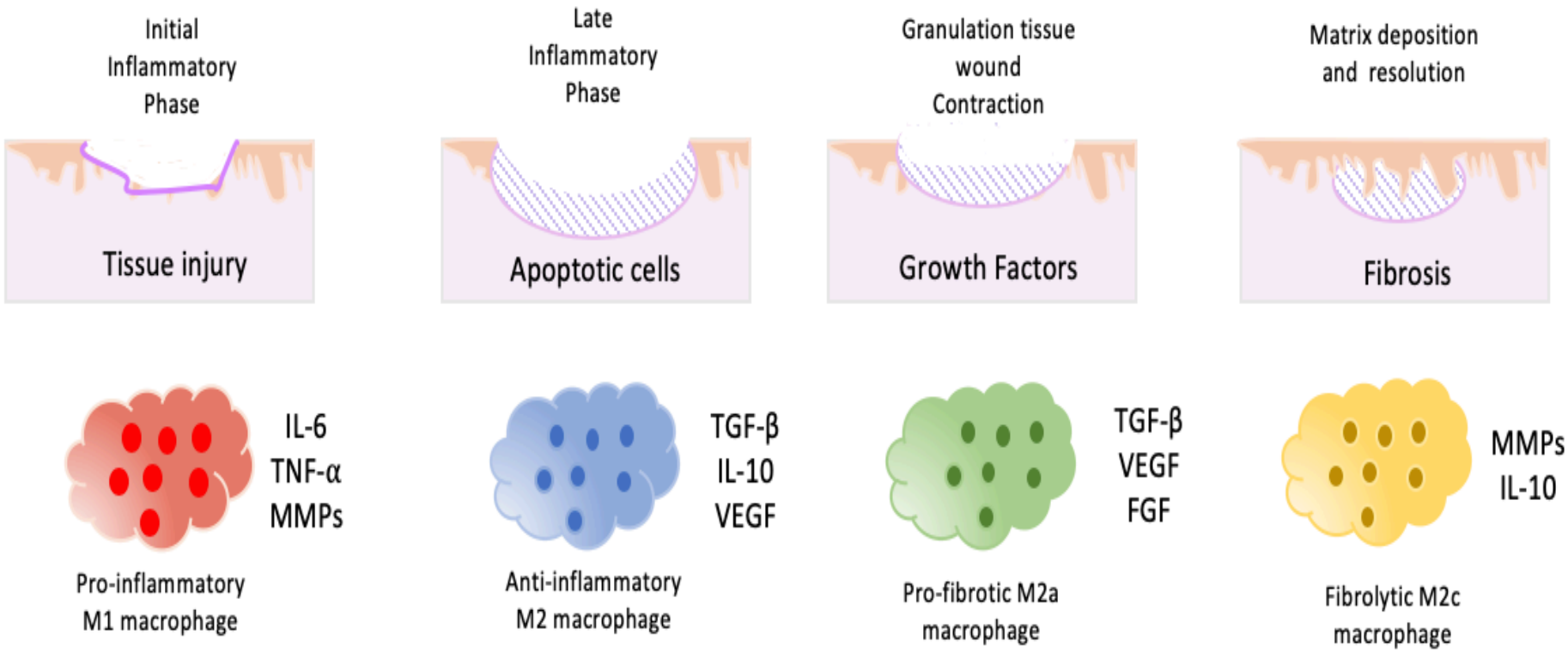


Fig 1.9. Two macrophages subtypes. As previously described, the macrophage subclassification is not only theoretical but also practical. As shown in figure the two different macrophage subtypes perform separate functions and activities during the wound healing process. Adapted from: Sindrilaru A, Scharffetter-Kochanek K. Disclosure of the Culprits: Macrophages—Versatile Regulators of Wound Healing. Advances in Wound Care. 2013;2:357-68.

1.2.3 The contradictory effects of nCRP and mCRP on monocytes and macrophages.

As aforementioned, an accumulation of monocytes/macrophages, nCRP and mCRP has been confirmed in several inflamed tissues. As aforementioned in paragraph 1.1.7.9, several studies have demonstrated that nCRP exert both anti and pro-inflammatory activities in a context dependant manner, whilst mCRP primarily encourages pro-inflammatory actions on different cell types, such as neutrophils, platelets and ECs. Similar results were also confirmed in both monocytes and macrophages. Eisenhardt et al. (2009b) reported that in monocytes, contrary to nCRP, mCRP binding through the lipid rafts and FCγRs can *(I)* activate the phosphatidylinositol 3-kinase (PI3K) intrinsic pathway; *(II)* increase monocyte adhesion and *(III)* enhance pro-inflammatory activity. In 2011, Eisenhardt et al. (2011), showed that mCRP prompts an exclusive release of pro-inflammatory markers in THP-1 monocytes. Similar results were also reported in others studies which confirmed that if M0 resting macrophages are stimulated with mCRP, they polarize into an M1 pro-inflammatory phenotype (Trial et al., 2016), whilst if stimulated by nCRP they polarize into an M2 anti-inflammatory phenotype, which induces the release of the IL-10 anti-inflammatory cytokine (Moore et al., 2001; Pilling et al., 2017). More recently, Sproston et al. (2018) confirmed that although mCRP is able to raise the inducible NOS (iNOS) and Nitric Oxide (NO) concentration in monocytes/macrophages, the same study also reported that nCRP reduces this release in the same type of cells. Therefore, observing the contrasting results, it seems that nCRP-mCRP dissociation is *the culprit* responsible for the pro-inflammatory activity even when the mCRP form binds to monocytes and/or macrophages (Wu et al., 2015). Furthermore, knowing that accumulation of both monocytes, macrophages and mCRP have been observed in several inflamed tissues and have been associated to some NCDs, such an IS (Slevin et al., 2010; Chiba and Umegaki, 2013), Alzheimer (Feng et al., 2011; Slevin et al., 2017; Mammana et al., 2018), CKD (Schwedler et al., 2003; Guiteras et al., 2016; Ohara et al., 2019) and infarcted myocardial tissue (Nahrendorf et al., 2010; Thiele et al., 2014b; O'Rourke et al., 2019), it has been postulated that the use of a small molecular inhibitor (SMI) may well

provide a useful strategy to block the potential mCRP pro-inflammatory (Thiele et al., 2015; Caprio et al., 2018).

1.3 Pharmacological treatment to reduce nCRP-mCRP dissociation and pro-inflammatory activity.

1.3.1 The use of nCRP-mCRP dissociation inhibitor may be a valid therapeutic strategy to block the mCRP pro-inflammatory activity.

Over the years, several studies have reported that the two CRPs (nCRP and mCRP) proteins exert opposing actions in several cell types (Sproston et al., 2018; Molins et al., 2008; Khreiss et al., 2004b). These contradictory results suggest that nCRP-mCRP dissociation is the underlying cause of pro-inflammatory activity (Thiele et al., 2015) and it has been postulated that the use of a small molecule inhibitor (SMI) may inhibit this process, making it a promising anti-inflammatory strategy (Thiele et al., 2015; Caprio et al., 2018). Pepys et al. (2006) documented that 1,6-bis(phosphocholine)-hexane (1,6-bis-PC) block the nCRP negative-activity by binding together two nCRP molecules. Later Thiele et al. (2014a) reported that the use of 1,6-bis-PC prevents dissociation from nCRP to mCRP, reducing the leukocyte recruitment and the TNF-α and IL-6 pro-inflammatory cytokine expression. However, although 1,6-bis-PC could be considered a potential nCRP-mCRP inhibitor (Pepys et al., 2006), literature also suggests that there are some limitations in the use of this SMI due to its short half-life and low affinity binding to the nCRP form (K_d= 150 nM) (Thiele et al., 2015). This limitation leaves open the opportunity to study other small molecules. Amongst which, the neurotransmitter Acetylcholine (ACh) appears to have a similar chemical structure to 1,6-bis-PC, which could be useful to block the mCRP pro-inflammatory activity more efficiently (Slevin et al., 2018). This hypothesis is further supported by the fact that, during the inflammation stage, the nCRP is known to reduce the serum-ACh levels (Nazarov et al., 2007) released through the cholinergic anti-inflammatory pathway (CAIP) (Murray and Reardon, 2018). This CAIP is an unusual anti-inflammatory mechanism activated by the tenth cranial nerve (Borovikova et al., 2000; Tracey, 2009).

1.3.2 The 10 cranial nerve.

The tenth cranial *vagus nerve* (VN) belongs to the autonomic nervous system (ANS) (Dantzer et al., 2000). This “wandering” nerve, connects to multiple organs and stimulates parasympathetic activity that is able to contrast the sympathetic activity induced by splanchnic nerves (Breit et al., 2018). Indeed, this cranial nerve innervates organs belonging to the cardiovascular and respiratory apparatus (Berthoud and Neuhuber, 2000), also regulate the heart and respiratory rate (Babic and Browning, 2014). Furthermore, this nerve is the main component of the neuroendocrine–immune axis and plays a pivotal anti-inflammatory role in infection and/or other inflammatory events (Tracey, 2007; Johnston and Webster, 2009). This anti-inflammatory activity is recognized as the inflammatory reflex (IR) (Andersson, 2005).

1.3.3 The inflammatory reflex.

The IR starts when PAMPs or DAMPs bind to the TLRs expressed on several immune systems (Kawasaki and Kawai, 2014; Hug et al., 2018) favouring the release of several pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α (Dantzer et al., 2000). Once these pro-inflammatory cytokines bind to their respective receptor present on VN-afferents fibres (Goehler et al., 1999; Tracey, 2002; Tracey, 2007; Oke and Tracey, 2008; Pavlov and Tracey, 2012), the VN-afferent fibers send an action potential to the nucleus tractus solitarius (NTS) localized in the medulla oblongata (Tracey, 2002). The NTS that connects to the corticotrophin-releasing-factor (CRF)-producing neurons present in the hypothalamus (Aguilera and Liu, 2012), stimulates the Hypothalamic–pituitary–adrenal (HPA) axis (Yan et al., 1998; Eskandari and Sternberg, 2002). The HPA send an action potential to both the nucleus ambiguus (NA) and the dorsal motor nucleus (DMN) (Bernik et al., 2002). The VN-effert fibres rise from the NA and DMN (**Fig 1.10**) and innervate various organs, such as the heart, lung, liver, intestine and celiac ganglion (Tracey, 2009; Howland, 2014; Yuan and Silberstein, 2016).

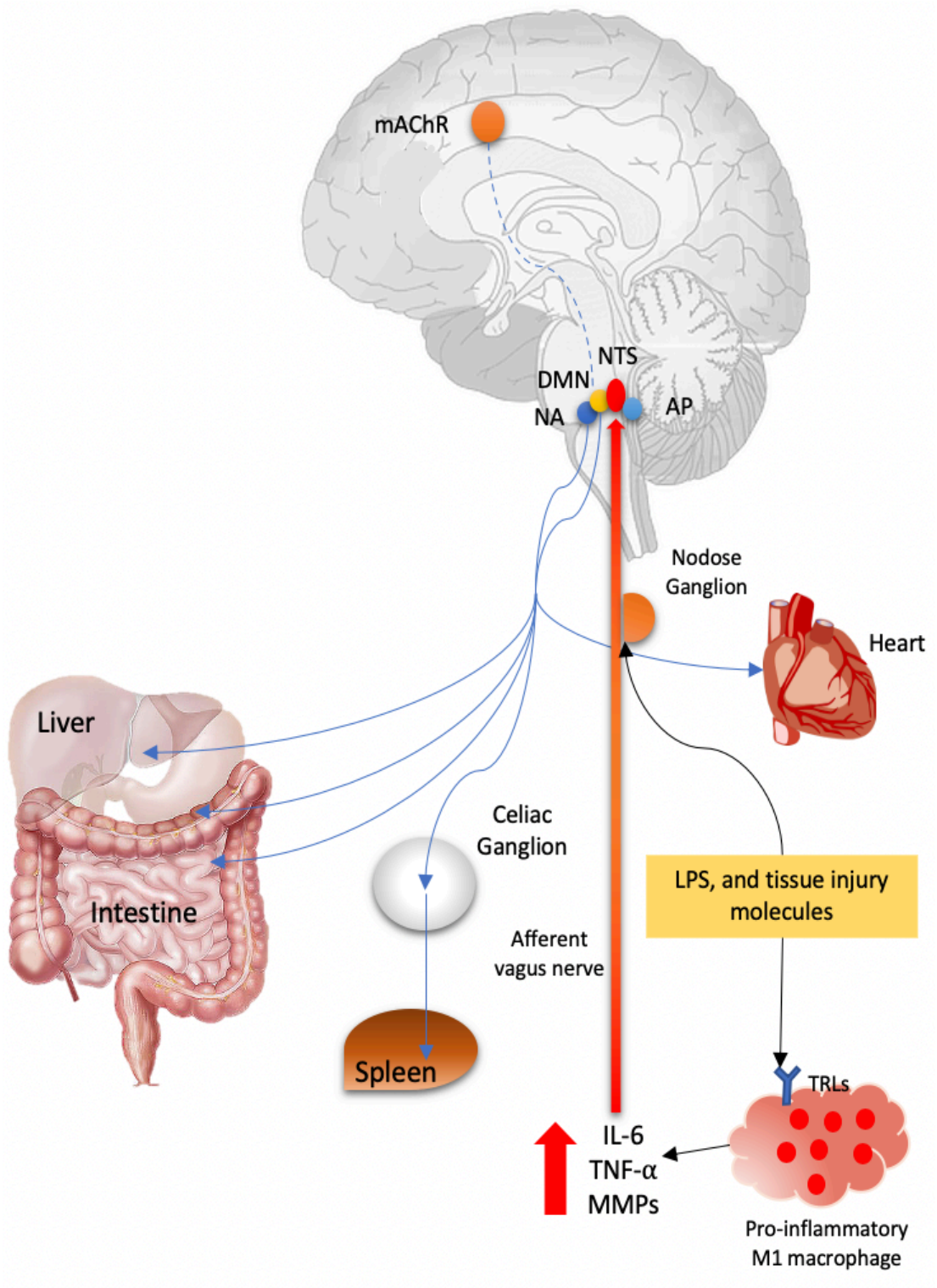


Fig 1.10. The inflammatory reflex. Immune cells release a large amount of cytokines when they detect LPS, pathogen fragments and/or tissue injury molecules. These cytokines activate the nucleus tractus solitaries (NTS) when binding with the VN sensitive afferent fibers (activated red line). The NTS connecting to the nucleus ambiguous (NA), the dorsal motor nucleus (DMN) and the area postrema (AP) is able to, activate the vagus nerves efferents (blue line). The VN cholinergic efferents then reduce the pro-inflammatory cytokine release through spleen, liver and gastrointestinal activity. Adapted Source: Pavlov VA, Tracey KJ. The vagus nerve and the inflammatory reflex - Linking immunity and metabolism. Nature Reviews Endocrinology. 2012;8(12):743-54.

1.3.4 The cholinergic anti-inflammatory pathway (CAIP), ACh and the α7 nicotinic acetylcholine receptor (α7nAChR).

As show in **Fig 1.11**, CAIP is activated when the VN-efferent fibre promotes the secretion of ACh into the celiac ganglion, which, in turn provokes the release of norepinephrine (NE) (also known as noradrenaline -NA-) into the spleen (Tracey, 2009). There the NE binds through β_2A receptors (β_2AR) expressed on $CD4^+$ $CD44^{high}CD62L^{low}$ T-cells stimulate the release of ACh (Pavlov and Tracey, 2005; Tracey, 2009; Wu et al., 2014). The ACh binds to the α7 nicotinic acetylcholine receptor (α7nAChR) on the macrophages (Orr-Urtreger et al., 1997; Wang et al., 2003; Gallowitsch-Puerta and Pavlov, 2007). The α7nAChR acts on JAK2, STAT3 and NF-KB transciptor factors **(Fig 1.11)** and blocks the pro-inflammatory cytokine release (Nathan, 2002; Tracey, 2002; de Jonge et al., 2005; Metz and Tracey, 2005; Tracey, 2007; Hoover, 2017; Li et al., 2018; Liu et al., 2018b; Yamada and Ichinose, 2018).

The α7nAChR sub-unit can also be activated by Nicotine which, a natural plant element with surprising anti-inflammatory activity (Kalra et al., 2004; Benowitz, 2009). It has been reported that when this compound binds to the α7nAChR, it blocks the NF-KB transcriptional activity also reducing the TNF-α pro-inflammatory cytokine release in U937 monocytes (Yoshikawa et al., 2006a). Tsoyi et al. (2011) reported that when Nicotine binds to α7nAChR it stimulates the PI3K/AkT/Nrf-2 intrisic pathway, which exerts an anti-inflammatory activity through the Heme Oxygenase-1 (HO-1) anti-inflammatory gene. More recently, St-Pierre et al. (2016) stated that when Nicotine binds to α7 and α9 nAChRs, it is able to reduce the ratio of pro-inflammatory versus anti-inflammatory monocytes with a consequent decrease in the liberation of pro-inflammatory cytokines.

1.2.1 The Anti-inflammatory activity of both ACh and Nicotine.

Due to their potential bond with the α7nAChR, both ACh and Nicotine have been protagonists of a series of studies that discussed their promising roles

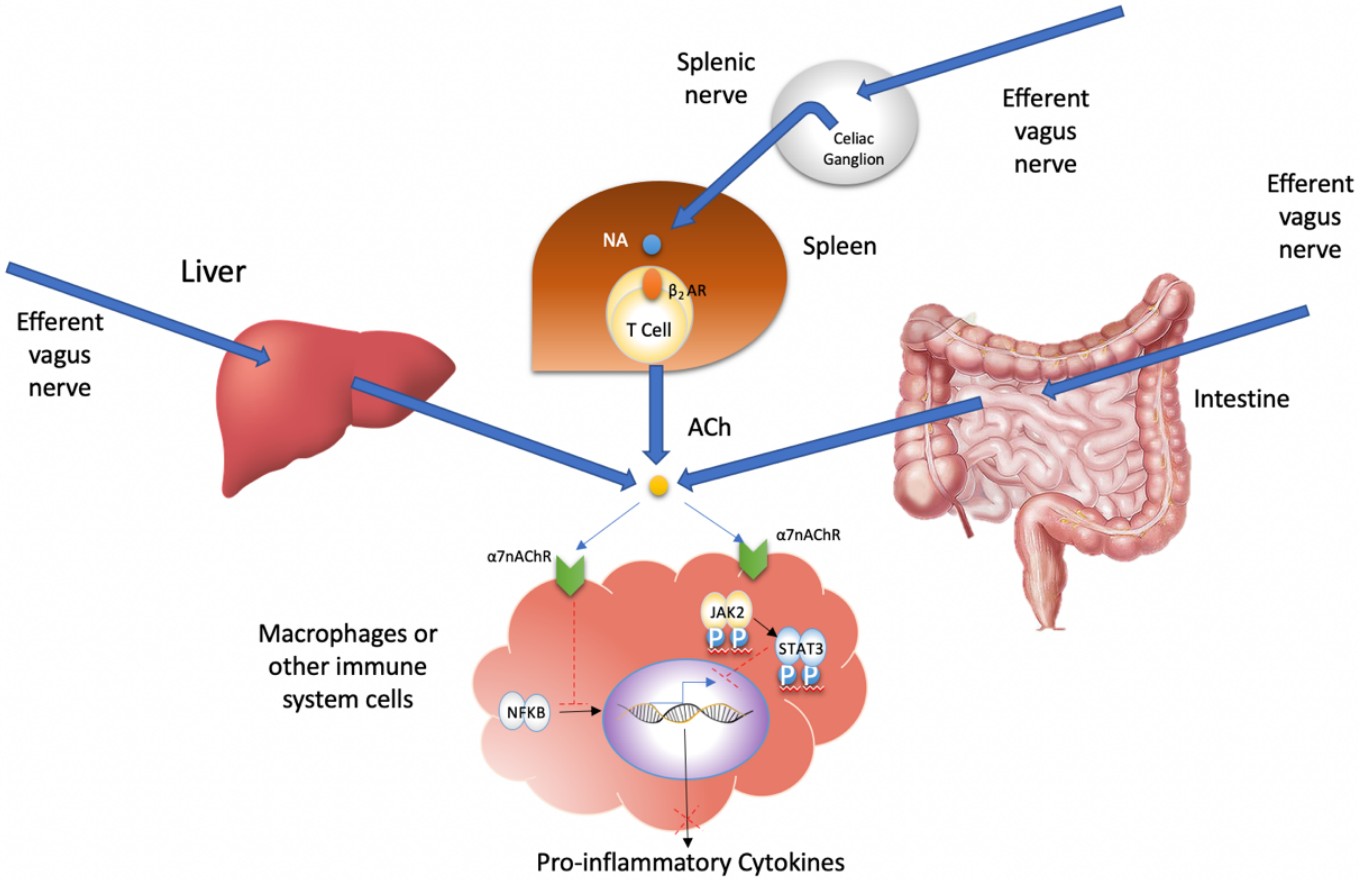


Fig 1.11. The protective effect of α7nAChR subunit. As aforementioned, the efferent VN increases the spleen, liver and gastrointestinal activity. These three organs increase the secretion of ACh. The anti-inflammatory activity is triggered when ACh binds to the α7 nicotinic acetylcholine receptor (α7nAChR) (expressed on macrophages or other immune cells) and is able to regulate the NF-KB and STAT3 activity reducing the pro-inflammatory cytokine release. Adapted from: Pavlov VA, Tracey KJ. The vagus nerve and the inflammatory reflex - Linking immunity and metabolism. Nature Reviews Endocrinology. 2012;8(12):743-54

as an anti-inflammatory therapeutic approach against several NCDs. Indeed, Caravaca et al. (2020) reported that the activation of CAIP could be a useful anti-inflammatory strategy against atherosclerosis and two other recent publications evidenced that both ACh and Nicotine have a potential anti-inflammatory activity against stroke and AD (Han et al., 2017; Katsuki and Matsumoto, 2018).

The mCRP presence and pro-inflammatory activity has been confirmed in these three NCDs. For example, Eisenhardt et al. (2009b) reported that the development of atherosclerosis is favoured when mCRP binds to monocytes through lipid rafts and FCγRs inside atherosclerotic plaques. Thiele et al. (2014b) reported the presence of mCRP in post-ischemic myocardium tissue and that it was associated to: *(I)* the increased expression of $CD68^{+}$; *(II)* to the enhanced adhesion of monocytes and ROS generation; and *(III)* triggered its pro-inflammatory activity when it bound to both CD64 (FCγI) and CD16 (FCγIII) receptors. Slevin et al. (2010) reported observing mCRP near newly formed blood vessels (angiogenesis) in IS tissue. Later, other studies demonstrated a clear link between mCRP accumulation in post-ischemic brain tissue and the development of AD dementia (Strang et al., 2012; Slevin et al., 2015; Slevin et al., 2017), even if the underlying mechanisms are still a question of debate.

Noteworthy is the fact that the observation of mCRP in all these NCDs has always been co-associated with the presence, recruitment and/or activation of both monocytes and macrophages (Eisenhardt et al., 2009b; Chiba and Umegaki, 2013; Thiele et al., 2014b; Thiele et al., 2015; Guiteras et al., 2016; Mammana et al., 2018), which are the same immune system cells that trigger a strong anti-inflammatory activity when stimulated by ACh and/or Nicotine through α7nAChR (Orr-Urtreger et al., 1997; Borovikova et al., 2000; Wang et al., 2003; Gallowitsch-Puerta and Pavlov, 2007; Benowitz, 2009; Han et al., 2017).

1.3 Aims

The aim of this project was to determine the effects of the mCRP form specifically expressed in several NCDs and chronic inflamed tissue. Therefore, the study carried out during this PhD project were focused on the mCRP-induced pro-

inflammatory cytokine production in human-derived U937 monocytes/macrophages, as an in-vitro model of inflammation.

1.4 Objectives

The specific objectives of the project were:

- To highlight whether the nCRP/mCRP dissociation could effectively be carried out experimentally.
- To evaluate whether the SMIs (ACh o Nicotine) are able to bind to nCRP and to inhibits its dissociation.
- To investigate the effects of exogenous exposure of mCRP isoforms on U937monocyte-derived macrophages.
- To evaluated whether the MAPK and NF-KB intrinsic pathways could be activated in U937monocyte-derived macrophages by mCRP stimulation.
- To evaluate the activity of the two cholinergic small molecular compounds, i.e., ACh and Nicotine, in order to assess their anti-inflammatory capacity.
- To investigate the α7nAChR role in reducing the human-derived U937 macrophage pro-inflammatory cytokine release.

CHAPTER 2 GENERAL MATERIALS AND METHODS.

2.1 Ethics.

Ethical approval for the *in vitro* study was granted by Manchester Metropolitan University (MMU) in strict agreement with the MMU Academic Ethics committee.

2.2 Materials.

Table 2.1 List of cell culture materials

Cell Culture	Supplier	Cat. Number
Human haematopoietic cell line U937	ATTC®	CRL-1593.2™
RPMI (RPMI 1640 with L-glutamine Lonza)	Lonza	BE12-702F
Fetal Bovine Serum -	Sigma®	FBS- F9665
Nunc EasYFlask 25cm2 T-25	Thermo-Fiscer scientific	156340
Nunc EasYFlask 75cm T-75	Thermo-Fisher scientific	156499 ·
6 well plate Nunclon™ Delta Surface	Thermo-Fisher scientific	140675 LOT158007
Tipone® 1000 µL	StarLab	S1122-1730
Tipone® 200 µL 1120-8710	StarLab	S1120-8710
Tipone®20/10 µL	StarLab	S1120-3710
Dulbecco's Phosphate Buffered Saline DPBS -1X-,9,5mM PO4 without Calcium or Magnesium, 500mL	Lonza	BE17-512F

Table 2.2 List of Antibodies and Primers

Antibodies and Primers	Supplier	Cat. Number
Anti–Hu CD11c	Thermo-Fisher scientific	LOT2008210
CD16 antibody	Abcam	[MEM-154] – Azide Free ab46679)
CD32 antibody	Abcam	[OTI9C6] ab124408)
CD64 antibody	Abcam	[10.1]–Low Endotoxin Azide Free ab185738)
Anti-NF-KB	Abcam	AB194758 phospho S529
Phospho-p38 MAPK	Cell Signaling Tecnology®	(Thr180/Tyr182) (28B10) Mouse mAb #9216
Secondary antibody Goat Anti-rabbit IgG (H+L) (human IgG-adsorbed) horseradish peroxidase conjugate	BioRad	170-6515
18S TaqMan® Gene Expression Assays	Thermo-Fisher scientific	Hs03003631_g1 4453320 Dye FAM-MGB
TNF TaqMan® Gene Expression Assays	Thermo-Fisher scientific	Hs00174128_m1 n˚: 4331182 Dye FAM-MGB
IL-6 TaqMan® Gene Expression	Thermo-Fisher scientific	Hs00174131_m1 n˚: 4331182 Dye FAM-MGB
IL-10 TaqMan® Gene Expression Assays	Thermo-Fisher scientific	Hs00961622_m1 Cat n˚: 4331182 Dye FAM-MGB

Table 2. 3 List of Manufactures' kits and reagents

Manufactures' kits and reagents	Supplier	Cat. Number
High Capacity Endotoxin Removal Spin Columns	Thermo Scientific™	88274
FITC Annexin V Apoptosis Detection Kit 1	BD Bioscience	556547 Lot 8072965
Alamar Blu	Cell Titer-Blue® Promega	G8080
BCA assay method (Pierce™ BCA Protein Assay Kit).	Thermo Scientific™	23227
TGX Stain-Free™ FastCast™ Acrylamide Kit, 12% from BioRad.	BioRad.	1610185
DuoSet ELISA Ancillary Reagent Kit 2	R&D Systems, bio-techbrand®.	DY008
Human TNF-α DuoSet ELISA	R&D Systems, bio-techbrand®.	DY210
Human IL-1β/IL-1F2 DuoSet ELISA	R&D Systems, bio-techbrand®.	DY201-05
Human IL-6 DuoSet ELISA	R&D Systems, bio-techbrand®.	DY206-05
Human CCL2/MCP-1	R&D Systems, bio-techbrand®.	DY27905
Human IFN-γ DuoSet ELISA	R&D Systems, bio-techbrand®.	DY285-05
Human IL-10 DuoSet ELISA	R&D Systems, bio-techbrand®.	DY217B-05
ECL Western Blotting Substrate Chemiluminescent Western ECL HRP	Thermo Scientific™Pierce™	32106
Kit Purification RNA (RNeasy plus)	Qiagen	74134
TaqMan Reverse Transcription reagents Applied Biosystems ® By Life Technologies ™	Thermo Scientific™Pierce™	1877894 REF N8080234
TaqMan™ Fast Advanced Master Mix	Applied Biosystems™	4444557

Table 2.4 List of Chemicals

Chemicals	Supplier	Cat. Number
Dimethyl sulfoxide	Sigma®	(DMSO 276855)
Trypan Blu solution	Corning®	17416001
Phorbol-12-myristate 13-acetate (PMA)	Sigma®	793461
Trypsin-EDTA solution Trypsin-EDTA (0.25%), phenol red)	Thermo-Scientific Gibco™	25200056
DAPI 4',6-Diamidine-2'-phenylindole dihydroch	Sigma®	D9542-1MG
Paraformaldehyde	Sigma®	P6148 LOT#041M1792V
Radioimmunoprecipitation (RIPA) buffer	Sigma®	R0278
Protease Inhibitor Cocktail (P8340 Sigma®)	Sigma®	P8340
Phosphatase Inhibitor Cocktail 2 (Sigma®).	Sigma®	P5726
N.N.N',N' Tetramethyyl-Ethylenediamine (TEMED) 100 ml Sigma®	Sigma®	LOT71K1515 T8133
Ammonium Persulfate (APS) A3678-25 LOT# MKBX2380V Sigma®	Sigma®	A3678-25 LOT# MKBX2380V
β-mercaptoethanol (2-Mercaptoethanol M3148-25ml Sigma®)	Sigma®	LOT#BCBH6010V
Tris-Base (TBS) LOT168464 BNP152-1 BP152-1	Fisher Bioreagents®	LOT168464 BNP152-1

Table 2.5 List of Chemicals

Chemicals	Supplier	Cat. Number
Tris-Base (TBS) LOT168464 BNP152-1 BP152-1	Fisher Bioreagents®	LOT168464 BNP152-1
Tween® 93773-250G	Sigma®	LOT#BCBF5959V
(Ethylenedinitrilo)tetraacetic acid (Edetic acid REF1233508-200MG Sigma®)	Sigma®	REF1233508-200MG
Triton-X 100 (Triton™ X-100 Sigma ®)	Sigma®	2315025
Invitrogen™ UltraPure™ Urea	Invitrogen™	15505027
Methylated Spirit (IDA 99), 99% (v/v), Pure, (Industrial Methylated Spirit, 74 0.P.)– Fischer Chemical	Fischer Chemical	10552904
Ethanolo (Ethanol 99%+, Absolute, Extra Pure, SLR, Fisher Chemical)	Fischer Chemical	10680993
Acetylcholine chloride (-ACh- 2-acetyloxyethyl(trimethyl)azanium; chloride	Sigma Aldrich®	A2661
Nicotine 3-[(2S)-1-methylpyrrolidin-2-yl]pyridine ((−)-Nicotine); Sigma Aldrich® Product Number N3876.	Sigma Aldrich	N3876.
-Tacrine- 1,2,3,4-tetrahydroacridin-9 amine;hydrochloride (Tetrahydroaminacrine hydrochloride hydrate-Tacrine-)	Sigma Aldrich	A79922

Table 2.6 List of other general materials

Other general materials	Supplier	Cat. Number
Lipopolysaccharides (LPS)	Sigma Aldrich	L2637
Nitrocellulose membranes	(Amersham Protran 0.45 NC nitrocellulose Western blotting membranes)	10600007
Slide-A-Lyzer™ Dialysis Cassettes	Thermo Fisher	66212
Amicon® Ultra centrifuge filter	Merck Millipore LtD	C7719
96 well black plate	(Greiner Bio One International	GmbH-REF655086 LOTE17123C9)
Falcon Tube for Flow cytometry	(Falcon™ Round-Bottom Polystyrene Tubes	Falcon 352058
Tipone®1.5 mL natural flat cap micro-centrifuge tubes	StarLab	Cat No S1615-5500
Tipone®2 mL	Natural flat cap micro-centrifuge tubes CP material	N°E1420-2000 LOT NOI 30717003
PCR Eppendorf (0.2 ml PCR Tube, Flat Cap –natural-)	StarLab	Cat. No. I1402-8100 LOT 18034)
Slide dual chamber for cell count	BioRad	Cat #145-0011
Centrifuge tube 15 ml (15ml, 120x17mm, PP)	Sarstedt Tube	NC9531248

Table 2.7. List of other general materials

Other general materials	Supplier	Cat. Number
Centrifuge tube 50 ml (Screw Cap Tube 50ml, 28X114mm, PP, conical base with skirted bottom)	Sarstedt Tube	NC9874179
Oxoid™ Phosphate Buffered Saline Tablets	Thermo Scientific™	Cat Num BR0014G
Pierce™ High Capacity Endotoxin Removal Spin Columns, 0.5 mL	Thermo Scientific™	2162373.3
Detoxi-Gel™ Endotoxin Removing Columns, 1 mL	Thermo Scientific™	Cat num 20344
Depc-Treatted Water, DNase/RNase free	Bioline	Cat No. BIO-38031
96-Well PCR Plate, Semi-Skirted, Raised Rim, Low Profile (for FAST® Systems), natural	Starlab	Cat. No. E1403-7700
C-reactive protein–Human- 5 mg-	Yo Protein	Cat Number 226
Eppendorf tube	(Greiner Bio-One Cryos™ 1 mL)	123278
Bovine Serum Albine	Sigma®	BSA A2153-100G LOT#SLBZ1221

Table 2.8 List of equipment

Equipment	Supplier	Cat. Number
Eppendorf centrifuge (Eppendorf Centrifuge 5415 D)	Sigma®	Z604062
Odyssey® Fc Imaging System	Licor	Not reported
Centrifuge Sigma 3-16K	DJB Lab care	10280
Spectrafuge™ 24D Digital Lab Microcentrifuge	Labnet international	C2400
-80 freezer (-86°C UltraFlow Freezer)	Nuaire	Not reported
Mr. Frosty (Mr. Frosty™ Freezing Container)	Thermo Scientific™	5100-0001
Water bath (Grant JB Nova)	Grant JB Nova	JBN12
Incubator (Nuaire ISO class 5 HEPA)	Nuaire	Not reported
Count cell (BioRad TC10™ automatic cell count)	BioRad	#1450010
Ice (Ice machine KF85)	Porkka	Not reported
Live cell imaging microscope	Leica	CTR 6000
FACS machine (BD FACSVerse™)	BD Bioscience	Not reported

Table 2.9 List of equipment

Equipment	Supplier	Cat. Number
Sonic machine (Grant Ultra sonic bath XUBA1)	The Laboratory Store	Not reported
Microplate Reader (Synergy™ HT Microplate Reader)	BioTek®	Not reported
Inc Accublock™ Digital Dry Bath	Labnet international	Not reported
PowerPac™ Basic	BioRad	#1645050
Electrophoresis unit	ATTO, Japan	AE-6500
Trans-blot SD Semydry Transfer Cell	BioRad	1703940
NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer	Thermo Scientific™	ND-ONE-W
Thermal cycler (Q-Cycler 96)	Hain Lifescience	7024007
Thermal cycler StepOnePlus™ Real-Time PCR System	Thermo Scientific™	4376600
dH_2O Elix® Progard TS2	Merck Millipore LtD	No PR0G0T0S2
Primo Vert Tissue Culture Microscope	ZEISS	Not reported
Mini Vortex Mixer Fisher Scientific	Fischer Scientifc	14-955-151

Table 2.10 List of Software

Zeiss ZEN Lite Software
Imagine Studio™ Lite (LI-COR®)
BDFacs suit V1.0.6
Microsoft Excel 2013 for Windows
Leica Application Suite X (LAS X) 3.4.2.18368
StepOne™ and StepOnePlus™ Software v2.3
GraphPad Prism version 7 for Windows GraphPad Software, La Jolla California USA (www.graphpad.com)

2.3 Monomeric C-reactive Protein (mCRP) preparation.

The pentameric or native (nCRP) commercial form was purchased from Yo protein laboratories (C-reactive protein –Human- 5 mg Cat Number 226 Yo Protein).

2.3.1 Dissociation from nCRP to mCRP.

The nCRP was inserted into a buffer containing 10 mM EDTA (Edetic acid REF1233508-200MG Sigma®) and 8 M urea (Invitrogen™ UltraPure™ Urea 15505027), to induce dissociation from nCRP into mCRP. The solution was incubated in a water bath (Grant JB Nova) at +37°C for two hours. This method was adopted in line with the Potempa protocol (Potempa et al., 1983; Potempa et al., 2015; Zhang et al., 2018).

2.3.2 Dialysis

After incubation, the nCRP was dialysed using Thermo Fisher Slide-A-Lyzer™ Dialysis Cassettes (Thermo Fisher Scientific). The mCRP was dialysed with a dH_2O buffer containing PBS (Thermo Scientific™ Oxoid™ Phosphate Buffered Saline Tablets Cat num BR0014G). Ten mL of mCRP was put into a 10 mL Fisher Slide-A-Lyzer™ G2 Dialysis Cassette. On day one the whole buffer (PBS + dH_2O + mCRP) was changed every two hours, for a total of three changes, using aseptic techniques. The mCRP was then maintained at a temperature of +4°C in

a dark room. After the third change, the mCRP was left to rest overnight at +4°C. The buffer was changed twice every four hours on day 2. After eight hours, the mCRP was recollected from the dialysis Cassette.

2.3.3 Protein Concentration

An Amicon® Ultra centrifuge filter (Amicon® Ultra centrifuge filter Merck Millipore LtD) was used to concentrate the dialysed protein, according to the manufacturer's instructions. The sample was loaded and the Amicon® Ultra centrifuge filter Merck Millipore LtD column was centrifuged (DJB Lab care Sigma 3-16K 10280) at 4500 x g for 25 minutes at +4°C.

2.3.4 mCRP Endotoxin Purification

A 0.5 mL Pierce™ High-Capacity Endotoxin Removal Spin Column was used to remove any endotoxin in the protein sample. The Endotoxin purification protocol was carried out according to the manufacturer's guidelines. Briefly, the column was put into a collection tube and spun at 500 × g for 1 minute so as to eliminate the storage solution. The column was then regenerated by 0.2N NaOH and inverted several times to ensure the resin was completely re-suspended in the solution and then incubated overnight at room temperature. The following day, the column was spun at 500 × g for 1 minute and the solution was discarded. A total of 2M NaCl was added to the column and inverted several times to ensure the resin was completely re-suspended in the mixture. The column was spun at 500 × g for 1 minute and the solution was discarded. Endotoxin-free ultrapure water was added to the column and the column was inverted several times to ensure the resin was suspended. The column was spun at 500 × g for 1 minute and the solution was discarded. The sample was added to the column and inverted several times to suspend the sample in the resin. The sample was incubated in the column at +4 °C overnight. The following day, the column was spun again (500 × g for 1 minute) and the sample was collected.

2.3.5 Anti-nCRP and anti-mCRP3H12 antibodies Endotoxin purification

Thermo Scientific™ Detoxi-Gel™ Endotoxin Removing Columns kit 1 mL (Cat num 20344) was used according to the manufacturer's instructions. Briefly, Detoxi-Gel Resin was degassed by suction for 15 minutes and the resin was left to precipitate for 30 minutes. The resin was washed it with sodium deoxycholate followed by pyrogen-free buffer. The sample was applied to the column in pyrogen-free buffer or water. The column flow was stopped for an hour once the sample had entered the resin bed, to optimise the yield. When the sample emerged from the column, it was collected.

2.4 Molecular Docking

All the SMIs were constructed and modelled, and energy minimized in neutral form by standard molecular mechanics methods. For the protein preparation, discovery studio Biovia 2020 (Dassault Systèmes, Vélizy-Villacoublay, France) which was use to visualize and modify receptor and ligand structures. Interaction analysis was carried out using the Accelrys Discovery Studio Visualizer. Atomic charges were assigned using the Gasteiger-Marsili procedure. For SMIs (docking with CRP subunit) the crystal structure of CRP was retrieved from the RCSB Protein Data Bank (PDB ID: 3PVO; resolution: 3.0 Å https://www.rcsb.org/structure/3PVO). The protein targets were refined using AutoDock 4.25 (Morris et al., 2009) using a well-established cross docking protocol to identify the conformations that have significant binding affinity with mCRP (Boraei et al., 2019). Briefly, water molecules were removed while hydrogen atoms were added using the ADT module implemented in AutoDock. The charges were adjusted using Gasteiger charge module for proteins implemented in AutoDock. Docking was performed for both complexes using the empirical free energy function and the Lamarckian protocol (Morris et al., 1998). The atomic charges for the protein were assigned using the same Gasteiger-Marsili method. Mass-centred grid maps were generated with 40 grid points for every direction and with 0.375 Å spacing by the AutoGrid program for the catalytic domains of CRP subunit. Starting position was randomly chosen on the entire protein surface as well as the orientations, and torsions were used for the ligands. The results were analysed for the presence of hydrogen bonding, hydrophobic

and p-p interactions between hits and the active site of the protein. The common interactions in both complexes were scrutinized. All the figures were rendered with the software Pymol and Jsmol.

2.5 Bicinchoninic Acid assay (BCA Assay).

Briefly, a 25 µL standard and sample was plated on 96-well microplate in duplicate (96-Well PCR Plate, Semi-Skirted, Raised Rim, Low Profile, for FAST® Systems, natural Cat. No. E1403-7700 Starlab). The BCA working solution was prepared by mixing 50 parts of reagent A and one part of reagent B (ratio 1:50). Afterwards, 200 µL of the final solution was added to each protein standard and sample. The plate was placed onto on a plate shaker delicately shaken for 30 seconds and incubated for 30 minutes at +37 °C. The optical densities were measured by a microplate reader (Synergy™ HT Microplate Reader BioTek®) at an absorbance of 562 nm after cooling the plate at room temperature for 5 minutes. The protein concentration was then calculated by standard curves. The results were calculated by the GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) and expressed in pg/mL.

Table 2.11 BCA Standards

Standards	Albumin concentration (µg/ml)
A	2000 µg/ml
B	1500 µg/ml
C	1000 µg/ml
D	750 µg/ml
E	500 µg/ml
F	250 µg/ml
G	125 µg/ml
H	25 µg/ml
I	0 µg/ml

2.6 Dot Blot Protein analysis.

Dot Blot was carried out using a total of 3 µL of media with 100 µg/mL of protein blotted onto nitrocellulose membranes (Amersham Protran 0.45 NC nitrocellulose

Western blotting membranes). The membranes were then blocked with 1% Bovine Serum Albumine (BSA - A2153-100G LOT#SLBZ1221 Sigma®) in TBS Tween PH 7.4 (Tris-Base –TBS- BNP152-1 BP152-1 Fisher Bioreagents® and Tween® 93773-250G Sigma®) for one hour, with gentle shaking, at room temperature. They were then incubated with anti-nCRP and anti-mCRP (anti-mCRP3H12) primary antibodies at +4°C overnight. After incubation, they were washed three times in TBS-Tween for ten minutes at room temperature. Afterwards, the membranes were incubated with the secondary antibody which was diluted 1:2000 with 5% milk (Marvel Dried Skimmed Milk Powder) and TBS-Tween for one hour, at room temperature. After a further washing cycle (three times, ten minutes each time, in TBS-Tween at room temperature), the chemiluminescent reagent was added to the membrane for one minute before developing the image. The membrane images were developed by the Odyssey® Fc Imaging System and analysed by the software Imagine Studio™ Lite (LI-COR).

2.7 General aseptic procedure.

All experiments and procedures were carried out in an aseptic environment under a class II biological safety cabinet (BSC) (WALKER safety cabinet limited), to avoid contamination by outside air. Before starting, the BSC was exposed to UV rays for at least 15 minutes followed by another 15 minutes of ongoing airflow and ethanol [70% volume/volume (v/v)] to sanitize the BSC surfaces. For good practice, only previously autoclaved (Dixon surgical LTD autoclave machine) sterile tips and other sterile materials were used. Before the experiments were set-up, the cells were controlled for any contamination under a microscope at x40 magnification (Primo Vert Tissue Culture Microscope ZEISS). Once these procedures had been completed, the BSC surface was cleaned again with 70% Industrial Methylated Spirit [-IMS Methylated Spirit IDA 99), 99% (v/v), Pure, (Industrial Methylated Spirit, 74 0.P.)–Fischer Chemical].

2.8 The human haematopoietic cell line U937.

The U937 monocyte cell line i.e. an oncogenic human monocyte cell line, differentiated into macrophage-like cells was used as an *in vitro* model of

inflammation. These cells had been harvested from a subject with generalised histiocytic lymphoma as reported by Sundström et al. (1976). In response to stimuli, they appeared to form into cells with the characteristics of macrophages (Passmore et al., 2001). These cells have also been used in other studies focused on the inflammation stage (Power et al., 2003; Yamagata et al., 2010; C. F. Vogel et al., 2012; Yamadera et al., 2018). They were also used to investigate mCRP production under conditions pertinent to the atherogenic process (Ciubotaru et al., 2005; Sproston et al., 2018).

General technique: As aforementioned, for safety reasons, all the experiments and procedures were carried out in an aseptic environment under a class II BSC according to the local bio-security and safety procedures. Firstly, the safety cabinet was sterilised with Ethanol [70% volume/volume (v/v)]. So as to reduce the possibility of contamination, only previously autoclaved sterile tips and other sterile materials were used. Furthermore, the growth medium (RPMI 1640 with L-glutamine Lonza, supplemented with 10% FBS -F9665 Sigma®-) was pre-warmed by placing a 50 mL centrifuge tube (Sarstedt Inc Screw Cap Tube 50 mL, 28X114mm, PP, conical base with skirted bottom) into a +37 °C water bath (Grant JB Nova) for at least 10 ten minutes, before starting treatment procedures.

2.9 Cryopreservation.

Fetal Bovine Serum (FBS F9665 Sigma®), was prepared with 10% Dimethyl sulfoxide (DMSO 276855 Sigma®) to complete the storage solution. Briefly, 1 mL of DMSO was mixed in 9 mL of cold FBS and stored at -20 °C until use. The growth medium was removed from the flask (T-25 Nunc™ Easyflask Thermo Scientific) and transferred to a 15 mL centrifuge tube (Sarstedt Tube 15ml, 120x17mm, PP). The tube was centrifuged at a speed of 1500 rpm (430 x g) for 7 minutes, then the growth medium was discarded. The storage solution was immediately added to the cell and aliquoted into 1 mL Eppendorf tubes (Greiner Bio-One Cryo.s™ 1 mL). After which, the Eppendorf tubes were put into a freezing container (Thermo Scientific™ Mr. Frosty™ Freezing Container), stored at -80°C (Nuaire -86°C UltraFlow Freezer) for 120 minutes, then removed from the freezer and stored in liquid nitrogen until use.

2.10 Cell recovery.

The Eppendorf tube was removed from the liquid nitrogen and immediately placed into a water bath at +37 °C for 2 minutes. The storage solution was then removed from the Eppendorf tube under the BSC and transferred to a 15 mL centrifuge tube. The tube was then centrifuged for 3 minutes at 2000 rpm (765 x g). The storage solution was then discarded and replaced with 2 mL of growth medium After pipetting, the growth medium was transferred into a T-25 flask and then incubated (ISO class 5 HEPA Nuaire) at + 37°C, 5% CO_2 95% air, humidified cell culture incubator.

2.11 Cell culture.

Monocyte U937 cells were grown in a growth medium (RPMI 1640 with L-glutamine Lonza) supplemented with 10% FBS (FBS F9665 Sigma®) in 95% humidified air with 5% CO_2 at +37°C in a T-75 flask (T-75 Nunc™ Easyflask Thermo Scientific) from passage 10 to 14.

2.11.1 Cell counting.

BioRad's TC10™ automated cell counter was used for cell count. A 20 µL cell suspension was extracted from the sub-cultured U937 monocyte cells and put into an Eppendorf tube containing 20 µL of trypan blue dye (Trypan Blu solution-Lot 17416001 Corning®). Ratio 1:1. After pipetting, 10 µL of the sample solution was loaded onto a dual-chamber slide (BioRad's Cell Counting Slide for TC10™ dual chamber Cat #145-0011). The slide was successively inserted into the TC10™ automated cell counter, which prompted the automatic cell count. Once this procedure was concluded, the machine showed the total cell count, the percentage of cell viability, and the final number of alive cells.

2.11.2 Passaging.

The media was removed from the flask (T-75 Nunc™ Easyflask Thermo Scientific) and transferred to a 50 mL centrifuge tube (Sarstedt Inc Screw Cap

Tube 50 mL, 28X114mm, PP, conical base with skirted bottom). The tube was centrifuged for 7 minutes at 1500 rpm (430 x g). After centrifuging, the spent growth medium was discarded and a new growth medium added in an amount that allowed for a cell concentration equal to 1 x 10^6 cell/mL. The growth medium was changed every three to four days.

2.12 Differentiation.

Literature reports that the monocyte U937 cell line has the potential to differentiate when treated with Phorbol-12-myristate 13-acetate (PMA) for 72 hours (Sproston et al., 2018). The following procedure was adopted in the present research. The spent growth medium was removed from the flask and put into a 50 mL centrifuge tube. The tube was centrifuged (DJB Lab care Sigma 3-16K 10280) for 7 minutes at 1,500 rpm (430 x g). After centrifuging, the spent growth medium was discarded and 3 mL of fresh growth medium was added. Cell viability and count were estimated by both the Trypan Blu solution and BioRad's TC10™ automated cell counter, as previously described. The initial cell viability was maintained above 90% for all experiments. The cells were then diluted at a 1 x 10^6 cell/mL concentration. The U937 cells were seeded at an initial density of 2 × 10^6 cell/well to induce to differentiation from floating monocytes into adherent macrophages. The differential medium contained RPMI 1640 with L-glutamine (1640 medium Lonza) supplemented with 10% FBS (F9665 Sigma®) and phorbol-12-myristate 13-acetate (PMA793461 Sigma®) at 50 ng/mL. The cells were grown on a 6-well plate (Nunclon™ Delta Surface Thermo-Scientific Cat. n°140675 LOT158007) for 72 hours. Following differentiation, the Dulbecco's Phosphate Buffered Saline (DPBS) was pre-warmed at +37°C in a water bath and then the cells were washed twice with the saline solution.

2.13 Enzyme-linked immunosorbent assays (ELISA).

Enzyme-Linked Immunosorbent Assay (ELISA) is a meticulous, rigorous, quantitative immunoassays method, which allows for the quantification of the antigen-antibody interaction through a catalytic action (Watanabe et al., 2013). As this technique has been widely used, it is not surprising to notice that multi-

analyses screening ELISAs have also been developed to detect simultaneous multiple antigen-antibody interactions (Zhang et al., 2011).

All the manufactures' kits and reagents were purchased by the R&D Systems bio-techbrand® and the tests were performed according to the manufacturer's instructions. To reduce the possibility of contamination, only previously autoclaved (Dixon surgical LTD autoclave machine) sterile tips and materials were used.

The medium was collected from a 6-well plate and inserted into an eppendorf tube and centrifuged at 1000 rpm (Eppendorf Centrifuge 5415 D) for 5 minutes. A total of 1800 µL of media was then transferred to another 2 mL eppendorf tube and stored at −80 °C (Nuaire -86°C UltraFlow Freezer) overnight. The captured antibody was diluted at working concentration in PBS (both capture antibody and PBS were included in the DuoSet ELISA Ancillary Reagent Kit 2 Catalog #DY008 R&D Systems bio-techbrand®). Following this step, the 96-well plate was coated with 100 µL of diluted capture antibody and incubated in a dark environment, at room temperature overnight. The day after the plate was washed 3 times with 400 µL wash buffer (washing buffer included in the same kit, i.e., the DuoSet ELISA Ancillary Reagent Kit 2). Once the washing cycle had been completed, the plate was blocked by adding 300 µL of reagent diluent (reagent diluent included in DuoSet ELISA Ancillary Reagent Kit 2) to each well. The plate was then incubated in a dark environment, at room temperature for a further 2 hours. Once these procedures had been completed, the plate was washed again 3 times with the washing buffer. After the last wash, 100 µL of the sample and standard curve (standard curve diluted in reagent diluent) was added to each well and incubated for 2 hours in a dark environment, at room temperature. All samples were added in duplicate. The plate was washed three more times with the washing buffer. A total of 100 µL of detection antibody (detection antibody included in the DuoSet ELISA Ancillary Reagent Kit 2) was added to the reagent diluent, then to each well and incubated in a dark environment, at room temperature for 2 hours. The plate was washed 3 more times with the same washing buffer and procedure. After the last wash, 100 µL of reagent diluents, containing Streptavidin-HRP (HRP

included in the DuoSet ELISA Ancillary Reagent Kit 2), was added to each well. The plate was covered and incubated for 20 minutes in a dark environment, at room temperature. After which, the plate was washed three more times and 100 µL of substrate solution (Solution A + Solution B in a ratio 1:1 included DuoSet ELISA Ancillary Reagent Kit 2) was added to each well and incubated for 20 minutes in a dark environment at room temperature. After 20 minutes 50 µL of stop solution (included in the DuoSet ELISA Ancillary Reagent Kit 2) was added to each well. The optical densities on the plates were read by a microplate reader at an absorbance of 450 nm. The protein concentration was then calculated by standard curves. The result was calculated by the GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) and expressed in pg/mL.

2.14 Cytotoxic testing.

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and Resazurin (element of Alamar Blue reagents) are two extremely useful compounds to determine if, organic extracts, nano-drugs, peptides, inhibitory RNA sequences and SMI exert a cytotoxic and/or anti-proliferative effect on cells (Ediriweera et al., 2018). However, there is a substantial difference between these two compounds. Differently to MTT, Alamar Blue is nontoxic to cells and provides results without killing them. Rampersad (2012) reported that Alamar Blue allows for a highly sensitive, user-friendly, non-toxic and non-radioactive assay that can potentially be used with both fluorescence and/or absorbance-based instrumentation readers.

In the experiments herein described, this cell assay was used to evaluate the level of the cytotoxic effect small molecule inhibitors (SMIs) have on U937-derived macrophages. Following differentiation (as aforementioned) the cells were treated for 24 hours by SMIs at different concentrations. Ethanol (Ethanol 99%+, Absolute, Extra Pure, SLR, Fisher Chemical) at 20% was used as a positive control. After 24 hours, Cell Titer-Blue® (Promega) was added and the medium was incubated (Nuaire ISO class 5 HEPA, 95% humidified air, with 5% CO_2, at +37°C) for 4 hours. Optical densities on plates were read by a microplate reader (Synergy™ HT Microplate Reader BioTek®) in the florescence mode

(530/25 excitation and 590/35 emission). The percentage of cell viability was calculated from the original raw data by Microsoft Excel 2013 for Windows. GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) was used for the graphs.

2.15 Apoptosis detection methods.

Macrophage apoptosis was estimated by the Annexin-V Fluorescein isothiocyanate (FITC) apoptosis detection kit (FITC Annexin V Apoptosis Detection Kit 1 Cat 556547 LOT8072965 BD Bioscience), according to the manufacturer's instructions. The cell samples were analysed by a BD FACSVerse™ (BD Bioscience) flow cytometry apparatus, with the aid of the BDFacs suit V1.0.6 software. This kit works as follows. Firstly, FITC Annexin V binding with phosphatidylserine (translocated during apoptosis) can be used to determine which percentage of cells are undergoing apoptosis. Secondly, Propidium Iodide (PI) is used to detect viable cells with undamaged membranes. This is possible because the membranes of dead and damaged cells are permeable to PI.

Following differentiation (as aforementioned), the compounds were added at a specific moment in time and at a specific concentration (the time-point and the specific concentration that had been previously identified by cytotoxic testing (2.15). After 24 hours in the incubator, the growth medium containing the compounds was removed from the 6 well plate. The cells were then washed with DPBS, which had been pre-warmed at +37°C in a water bath. After washing, the cells were trypsinized with Trypsin-EDTA solution and then incubated for 5 minutes. After this procedure, the cells were checked (Primo Vert Tissue Culture Microscope ZEISS), to confirm if the cells had completely detached. A total of 4.5 mL of RPMI 1640 with L-glutamine (1640 medium Lonza), supplemented with 10% of FBS (F9665 Sigma®) was added to the cells to block the Trypsin activity. The cells were then transferred to a centrifuge tube and centrifuged for 7 minutes at a speed equal to 1500 rpm (430 x g). After which, the solution (containing RPMI 1640 with L-glutamine, 10% FBS and Trypsin-EDTA solution) was removed. The

cells were washed twice with DPBS and resuspended in 1X binding buffer (containing FITC Annexin V Apoptosis Detection Kit 1 Cat 556547 LOT8072965 BD Bioscience) at a concentration of 1 x 10^6 cells/mL. 100 µL of the solution (1 x 10^5 cells) was then transferred to the falcon tube for flow cytometry (Falcon™ Round-Bottom Polystyrene Tubes Falcon 352058). Five µL of FITC Annexin V and 5 µL PI was then added to the tube which was then vortexed (Fisher Scientific Mini Vortex Mixer) for 30 seconds and incubated for 15 minutes, at room temperature, in a dark environment. Fifteen minutes later, 400 µL of 1X Binding Buffer was added to each tube and both BD FACSVerse™ (BD Bioscience) and BDFacs suit V1.0.6 software were used to analyze the apoptotic effect of each compound.

2.16 Flow cytometry.

Flow cytometry (FCM) is a technique used in the immunological field (Cossarizza et al., 2017) as it is able to identify several cellular features (including size and internal complexity) through the analysis of intermittent laser light (Henel and Schmitz, 2007).

Following differentiation (as previously described), the cells were washed three times with DPBS pre-warmed at +37°C in a water bath. A total of 1.5 mL of Trypsin-EDTA solution was added to the cell and the solution was incubated for 5 minutes. After this procedure an inverted phase contrast cell culture microscope, was used to confirm if the cells had completely detached (Primo Vert Tissue Culture Microscope ZEISS). A total of 4.5 mL of RPMI 1640 with L-glutamine (1640 medium Lonza), supplemented with 10% of FBS (F9665 Sigma®), were added to the cells to block the Trypsin activity. Following this procedure, the cells were transferred to a centrifuge tube and centrifuged for 7 minutes, at a speed of 1500 rpm (430 x g). Seven minutes later, the solution (containing RPMI 1640 with L-glutamine, 10% FBS and Trypsin-EDTA solution) was removed. The cells were then transferred from the 15 mL Sarstedt tube to a previously autoclaved eppendorf tube through DPBS. Following the transfer procedure, the cells were washed twice in DPBS and centrifuged (Eppendorf

Centrifuge 5415 D) at 500 x g for 5 minutes, at room temperature. After the second washing, the cells were suspend in a 200 µL solution of 4% paraformaldehyde (P6148-500G LOT#041M1792V Sigma®), dissolved in dH_2O and incubated in a dark environment, at room temperature for 15 minutes. After which, they were washed thoroughly with 1 mL DPBS and immediately centrifuged, at 500 x g for 5 minutes. Following the last washing procedure, 0.125 µg/mL of anti-human CD11c antibody (Thermo-fisher Anti–Hu CD11c LOT2008210) was diluted in a washing buffer containing 10% FBS (F9665 Sigma®) and DPBS (BE17-512F Lonza). The antibody was then added and incubated at +4°C in a dark chamber (fridge) for 30 minutes. A total of 200 µL of washing buffer was then added to the cells and centrifuged at 500 x g for 5 minutes. Once the last washing phase had been completed, the supernatant was removed and 500 µL of DPBS (BE17-512F Lonza) was added to transfer the cells into the flow cytometry tube (Falcon™ Round-Bottom Polystyrene Tubes Falcon 352058). The final data were obtained by the BD FACSVerse™ (BD Bioscience) and the analyses were carried out by the BDFacs suit V1.0.6 software.

2.17 Western Blots.

The first technique used to transfer the proteins from a polyacrylamide gel to a nitrocellulose sheet was described in 1979 (Towbin et al., 1979). Western blotting (WB), is a biological molecular technique (Mahmood and Yang, 2012) able to identify specific proteins once they have been separated using a vertical gel electrophoresis system, blotted onto nitrocellulose membranes and incubated with a selected antibody. WB is one of the most preferred quantification protein methods adopted in molecular biology laboratories.

So as to reduce the possibility of contamination, only previously autoclaved (Dixon surgical LTD autoclave machine) sterile tips and materials were used. The medium was collected from the 6-well plate and put into a 15 mL centrifuge tube. The cells were lysed by adding 200 µL/well of ice-cold buffer Radioimmunoprecipitation (RIPA) (R0278 Sigma®) containing a protease inhibitor cocktail (P8340 Sigma®) and a phosphatase inhibitor cocktail 2 (P5726

Sigma®) on ice (Ice machine Porkka KF85). The lysed cells were then put in an Eppendorf tube (Greiner Bio-One Cryos™ 1 mL), sonicated for 30 seconds (Grant Ultra sonic bath XUBA1) and centrifuged (Eppendorf Centrifuge 5415 D) at 0.1 rfc for one minute. After centrifugation, 180 µL of protein lysates was transferred into another eppendorf tube. The protein concentration of the lysed cells was determined by the BCA assay method (Pierce™ BCA Protein Assay Kit), as previously described. At the end of the BCA assay procedure, the sample was stored in a -80 °C in a freezer overnight. So as to reduce and denature the sample, the following day the sample was diluted onto a loading buffer (traditional laemmli buffer) containing β-mercaptoethanol (2-Mercaptoethanol M3148-25ml LOT#BCBH6010V Sigma®) and bromophenol blue. The samples were warmed for 5 minutes at a +95 °C (Inc Accublock ™ Digital Dry Bath Labnet international). Equal amounts of protein extracts were electrophoresed on 12% SDS polyacrylamide gels (TGX Stain-Free™ FastCast™ Acrylamide Kit 12% from Bio-Rad, TEMED Sigma® LOT71K1515 T8133 100 mL, Ammonium Persulfate –APS- A3678-25 Sigma® LOT# MKBX2380V). Once the electrophoresed had been completed, the phase proteins were electroblotted, using a semi-dry apparatus (Trans-blot SD Semydry Transfer Cell BioRad), onto a nitrocellulose membrane (Amersham Protran 0.45 NC nitrocellulose Western blotting membranes). The membranes were then blocked by adding 1% Bovine Serum Albine (BSA A2153-100G LOT#SLBZ1221 Sigma®), diluted in TBS Tween (Tris-Base –TBS- Fisher Bioreagents® LOT168464 BNP152-1 BP152-1; Tween® 93773-250G LOT#BCBF5959V Sigma® and dH_20 at pH 7.4). The membranes were shaken gently in a dark environment, at room temperature, for 1 hour. They were then stained with a primary antibody (Anti-NF-KBp65-phospho S529-antibody AB194758 Abcam; Phospho-p38 MAPK-Thr180/Tyr182-28B10- Mouse mAb#9216 Cell Signaling Tecnology®), which had been diluted in a blocking buffer containing 1% BSA in TBS-Tween (Ph 7.4). The membranes and the antibodies were then shaken gently in a dark environment, at +4 °C overnight. On the second day, the membranes were washed three times (10 minutes each time), with TBS-Tween in a dark environment, at room temperature. Once the last washing phase had been completed, the membranes were incubated again with

a horse-radish peroxidase-conjugated antibody (Secondary antibody Goat Anti-rabbit IgG -H+L-human IgG-adsorbed horseradish peroxidase conjugate -HRP-Cat n° 170-6515 BioRad) diluted in (1:3000) in TBS-Tween containing 5% of de-fatted milk (Marvel Dried Skimmed Milk Powder) in a dark environment, at room temperature, with continuous mixing for 1 hour. After which, they were washed three times (as described) in TBS Tween PH 7.4. The membrane was visualized using the ECL chemiluminescence detection method. Membrane images were obtained with the Odyssey® Fc Imaging System and were analysed by the software Imagine Studio™ Lite (LI-COR).

2.18 Reverse transcription-polymerase chain reaction (RT-PCR).

Polymerase chain reaction (PCR) is a technique able to amplify a few DNA segments into thousands of millions of copies, using a DNA-polymerase enzyme and a primer (Valones et al., 2009). As this is an extremely rapid, cost-effective and user-friendly technique, there is an increase in publications reporting its use (VanGuilder et al., 2008). In this investigation a specific type of qRT-PCR, the Reverse transcriptases-qPCR (RT-qPCR), was used.

2.18.1 RNA purification.

The RNA purification procedure in this study was carried out on ice. Moreover, only previously autoclaved sterile material was used.

The medium was collected from a 6-well plate and discharged a 15 mL centrifuge tube. Cell lysing was done by adding 350 µL of Buffer RLT Plus (the lysis buffer included the RNeasy plus mini kit 50 Cat No./ID:74134 Qiagen) containing β-mercaptoethanol. The lysed cells were collected and stored overnight at −80°C before starting the RNA purification procedure, which was carried out using a dedicated kit (RNeasy plus mini kit 50 Cat No./ID:74134 Qiagen).

After removal from storage the day after, the sample was transferred from the Eppendorf tube to the gDNA eliminator column (included in the RNeasy plus mini

kit). It was then centrifuged at more than ≥11,000 rpm for 30 seconds. The column was then disposed of and the flow-through was saved. A total of 350 µL of 70% ethanol was added and mixed to the column. Seven-hundred µL (350 µL of cell lyse + 350 µL of 70% ethanol) of the sample was then transferred to the RNeasy spin column and transferred to a 2 mL collection tube (both the RNeasy spin column and the collection tube were supplied in the kit) and centrifuged for 15 seconds at more than 11,000 rpm. The flow-through was once again disposed of. A further 500 µL of Buffer RW1 (buffer included in the RNeasy plus mini kit) was added to the RNeasy spin column and centrifuged for 15 seconds, at more than 11,000 rpm to wash the spin column. The flow-through was disposed of again. Five-hundred µL buffer RPE (buffer included in the RNeasy plus mini kit) was added to the RNeasy spin column and centrifuged for 15 seconds at ≥11,000 rpm to rinse the RNeasy spin column membrane. The RNeasy spin column was then relocated to a new 2 mL collection tube and both the old collection tube and the flow-through were disposed of. The new collection tube was centrifuged for 1 minute. The RNeasy spin column was transferred to a new 1.5 mL collection tube (supplied) and 30 µL of RNase-free water (RNase-free water included in the RNeasy plus mini kit) was directly added to the spin column membrane. Lastly, the RNeasy spin column was centrifuged for one minute at more than 11,000 rpm to clear the spin column membrane. The last two steps were repeated one more time.

2.18.2 RNA concentration phase.

In summary, 1 µL of each sample was loaded onto the Thermo Scientific™ NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer used to quantify the RNA concentration. Once this procedure had been concluded, the machine provided an automatic quantification of the total RNA concentration.

2.18.3 The reverse transcription phase.

The cDNA was obtained through the reverse transcription phase TaqMan Reverse Transcription reagents (LOT 1877894 REF N8080234 Applied Biosystems® By Life Technologies™). Briefly, an equal volume of 500 ng/mL of

RNA was transferred into a 0.2 mL PCR tube with2 µL of X10 RT Buffer, .4 µL of Magnesium chloride ($MgCl_2$), 1 µL of dNTP Mixture , 1 µL of RNase Inhibitor, 1 µL MultiScribe™ Reverse Transcriptase, 1 µL of Random Hexamers. The remaining super pure dH_2O (Depc-Treated Water, DNase/RNase free Cat n°. BIO-38031 Bioline) so as to arrive at a total volume of 20 µL. The mixed components were vortexed (Mini Vortex Mixer Fisher Scientific) and centrifuged (Spectrafuge™ 24D Digital Lab Microcentrifuge Labnet international) briefly to collect solutions at the bottom of the reaction tubes. The reaction mix was then incubated inside a Thermal cycler (Q-Cycler 96 Hain Lifescience) according to the protocol described in **Table 2.12.** Once the incubation phase had been completed, the mixed contents were vortexed again.

Table 2.12 The protocol for reverse transcription phase.

TEMPERATURE (˚C)	TIME (MINUTES)
+25°C	10
+37°C	30
+95°C	5
+4°C	HOLD

2.18.4 Real time PCR

This phase was performed using both the Applied Biosystems™ TaqMan™ Fast Advanced Master Mix and the TaqMan® Gene Expression Assays (18S; IL-1β; IL-6; IL-10). All the reagents were plated in 96-well PCR Plate in the appropriate quantity, as reported in **Table 2.13**.

Table 2.13 Quantity of reagent volume necessary for the PCR phase.

TaqMan™ Fast Advanced Master Mix	10 µL
TaqMan® Gene Expression Assays	1 µL
Depc-Treated Water, DNase/RNase free Cat No. BIO-38031 BIOLINE	7 µL
RT	2 µL

The plate was then centrifuged briefly to collect solutions at the bottom of the reaction tubes. StepOnePlus™ The Real-Time PCR System was used as a Thermal Cycler. The amplification protocol was performed according to the manufacturer's instructions and is summarised in **Table 2.14.**

Table 2.14 The PCR phase protocol

UNG Incubation	Polymerase Activation	PCR (40 cycles)	
Hold +50°C	Hold +95°C	Denature +95°C	Annealing / Extension +60°C
2 minutes	2 minutes	1 second	20 seconds

StepOne™ and StepOnePlus™ Software v2.3 extrapolated the results, while Microsoft Excel 2013 for Windows and GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) provided the data analysis.

2.19 Statistical Analyses.

GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) was used for all the statistical analyses. Statistical significance was set at a p-value of <0.05 and a 95% Confidence Interval (CI) was fixed, while errors were fixed at 5 %. The same software was also used to produce graphical outputs. Where appropriate, experiments were validated by the One-way analysis of variance (ANOVA) test, followed by Tukey's post-test or Student’s t-test.

CHAPTER 3 RESULTS: How Acetylcholine affects the dissociation of native C-Reactive Protein to monomeric CRP.

CHAPTER 3: How ACh affects the dissociation of nCRP to mCRP.

A persistently acute phase response (APR) can promote atherogenesis, atherosclerosis incidents (Rader, 2000; Lowenstein and Matsushita, 2004; Ambrose and Barua, 2004; Libby, 2012; Siasos et al., 2014; Poulsen et al., 2015; Katakami, 2018) and increase nCRP concentration (Ceron et al., 2005).

A study, carried out in 2009, reported that activated platelets were able to dissociate nCRP into monomeric C-reactive protein (mCRP) when these cells were found near the human aorta and carotid artery atherosclerotic plaques (Eisenhardt et al., 2009b). Once dissociated, mCRP acquires different functional properties to the original nCRP. Indeed, T. Khreiss et al. (2004) reported that mCRP was able to up-regulate P-selection and increase the formation of neutrophil-platelets and neutrophil aggregation when the monomer bounded with the FCγIIIb (CD16) receptors. Taylor and Van Den Berg (2007) reported that this new monomeric conformational form (mCRP), differently to nCRP, was also able to bind to human umbilical vein endothelial cells (HUVECs). Eisenhardt et al. (2009b) study described how this *in vivo* dissociation was followed by an increase in monocyte adhesion and Reactive Oxygen Species (ROS) but this process was abolished when CD64 (FCγI), CD32 (FCγIIa), CD16 (FCγIII) receptors were blocked. It has been observed that mCRP is able to increase paracellular permeability, lower the transepithelial electrical resistance and reduce ZO-1 expression, as well as occluding proteins, when mCRP binds with retinal pigment epithelial cells (Molins et al., 2017). A recent review has evidenced that, while nCRP is characterised by both pro and anti-inflammatory activities, mCRP mainly features pro-inflammatory proprieties on different cell types, such as endothelial cells leukocytes and platelets (Wu et al., 2015).

The contradictory literature data as to the role nCRP plays in inflammation posed a challenge for the estimation of exactly how much weight nCRP carries in the inflammatory response (Sproston and Ashworth, 2018). Thiele et al. (2015) stated that these pro-inflammatory features can now be imputed to the monomeric isoform and the prevention of the dissociation from nCRP to mCRP may well be a way to block pro-inflammatory activity. Consequently, some

authors have attempted to impair nCRP to mCRP dissociation with different approaches (Thiele et al., 2014a; Thiele et al., 2015).

A small molecular dissociate-inhibitor was used to block the nCRP and mCRP pro-inflammatory activity. Based on fact that Pepys et al. (2006) confirmed that 1,6-bis(phosphocholine)-hexane acts as a specific small-molecule inhibitor of nCRP activity, Thiele et al. (2014a), reported that mCRP triggered its pro-inflammatory activity when it bound to both CD64 (FCγI) and CD16 (FCγIII) receptors and the use of 1,6-bis-PC (inhibiting the dissociation from nCRP to mCRP) reduced leukocyte recruitment and TNF-α expression, as well as IL-6 pro-inflammatory cytokines release (Thiele et al., 2014a).

Several studies have recently reported that an increase in pro-inflammatory cytokines has been associated with an inflammatory reflex activation (Tracey, 2002; Tracey, 2007; Oke and Tracey, 2008; Pavlov and Tracey, 2012). Inflammatory reflex activation is associated with an increase in Acetylcholine (ACh) through the cholinergic anti-inflammatory pathway (CAIP) (Pavlov and Tracey, 2005; Tracey, 2009). Considering the close structural homology between 1,6-bis(phosphocholine)-hexane and the ACh neurotransmitter (Slevin et al., 2018), it is reasonable to hypothesize that ACh neurotransmitter might also bind to nCRP, inhibiting its dissociation. This hypothesis is further supported by the fact that nCRP is known to lead to lower ACh levels in plasma via a capture process (Nazarov et al., 2007). Therefore, there is the possibility that when the ACh neurotransmitter binds to nCRP, it acquires the capacity to prevent dissociation from nCRP into mCRP.

Furthermore, two other small cholinergic molecules, i.e., Nicotine and Tacrine, were also tested during this research to assess whether these compounds were able to bind to nCRP, thus inhibiting dissociation into mCRP.

Aim and objectives: To obtain further information on whether SMIs such as ACh, Tacrine and Nicotine can be used to block, or at least reduce, nCRP-mCRP dissociation.

This was addressed by the following objectives:

- The literature reports that preventing the dissociation from nCRP to mCRP may well be a way to block pro-inflammatory activity. However, the fact that there are two different CRP (nCRP and mCRP) forms makes it difficult to ensure a selective strategy. The use of anti-mCRP antibody could be potential strategy to address this issue. On the basis of this reasoning, a first experiment was designed to make a selective identification of the two different forms of CRP, by the use of antibodies. Several studies have used dissociation methods against nCRP, e.g., Potempa et al. (1983) and Taylor and Van Den Berg (2007). Both methods, i.e., Potempa's and heating method, were assessed to evaluate how the nCRP/mCRP ratio is affected during the dissociation phase.

- The 1,6-bis(phosphocholine)-hexane has a very similar chemical strucuture to that exerted by ACh (Slevin et al., 2018). Should other molecules be proven able to inhibit nCRP-mCRP dissociation, a new class of compounds that can be used against nCRP to mitigate its pro-inflammatory effect could be proposed. Therefore, the primary aim of this research was to determine whether selected Small Molecular Inhibitors (SMIs), i.e., ACh, Tacrine and Nicotine, have the capacity to block or impair nCRP-mCRP dissociation.

3.1 Antibody evaluation.

Therapeutic antibodies have been used to treat several diseases. The possibility to adopt this strategy against nCRP prompted the evaluation of the efficacy of Anti-nCRP in binding to the nCRP. The anti-mCRP3H12 mouse monoclonal antibody was developed by Dr L.A. Potempa and fully characterized as previously described (Diehl et al., 2000). The experiment was designed to assess the antibody binding selectivity against both the native CRP (nCRP) and the monomeric CRP (mCRP) forms, respectively (**Fig. 3.1).** Anti-nCRP and anti- showed a strong bind with both nCRP and mCRP respectively.

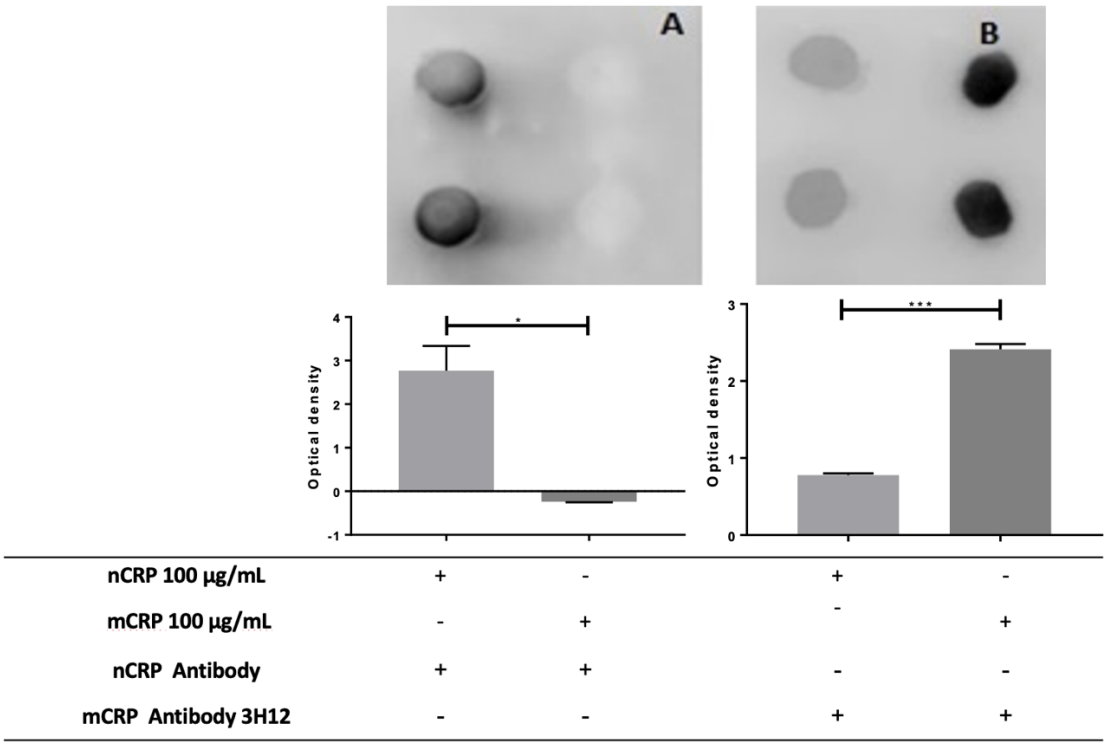


Fig. 3.1. **Antibody efficiency evaluation.** Anti-nCRP and anti-mCRP3H12 were characterized. Both showed a strong capacity to bind with both nCRP and mCRP respectively. In this dot-blot binding assay, 100 µg/mL nCRP and mCRP were loaded onto the membrane. The unpaired One-tailed t-test, with a 95% Confidence Interval (CI) and an α error fixed at 5% showed that, at the same concentration (100 µg/mL), the anti-nCRP antibody selectively bound with the nCRP obtained from Yo protein laboratories (p-value* <0.0086). At the same concentration (100 µg/mL) and using the same statistical parameters, the anti-mCRP3H12 antibody showed the same selectivity to bind with the appropriate conformational mCRP form (p-value ***<0.0005). (n=3)

3.2 The Dissociation method against native-CRP.

So as to determine the best way to dissociate nCRP from a pentameric to a monomeric, nCRP was incubated in high concentrations of urea (Potempa et al., 1983; Williams et al., 2020). Potempa et al. (1983) demonstrated that the pentameric nCRP form was dissociated into the monomeric form (mCRP) by 10 mM EDTA and 8 M urea incubated at +37°C for two hours.

As shown in **Fig. 3.2,** a strong dissociation from the nCRP form to the monomeric form (mCRP) was observed. Dialysis was then performed. However, it was not clear how this procedure is able to attenuate the capacity of anti-mCRP3H12 in binding with the mCRP form (**Fig. 3.2**)**.** It was observed that the two antibodies i.e, Anti-nCRP and anti-mCRP3H12 were able to bind both with nCRP and mCRP respectively.

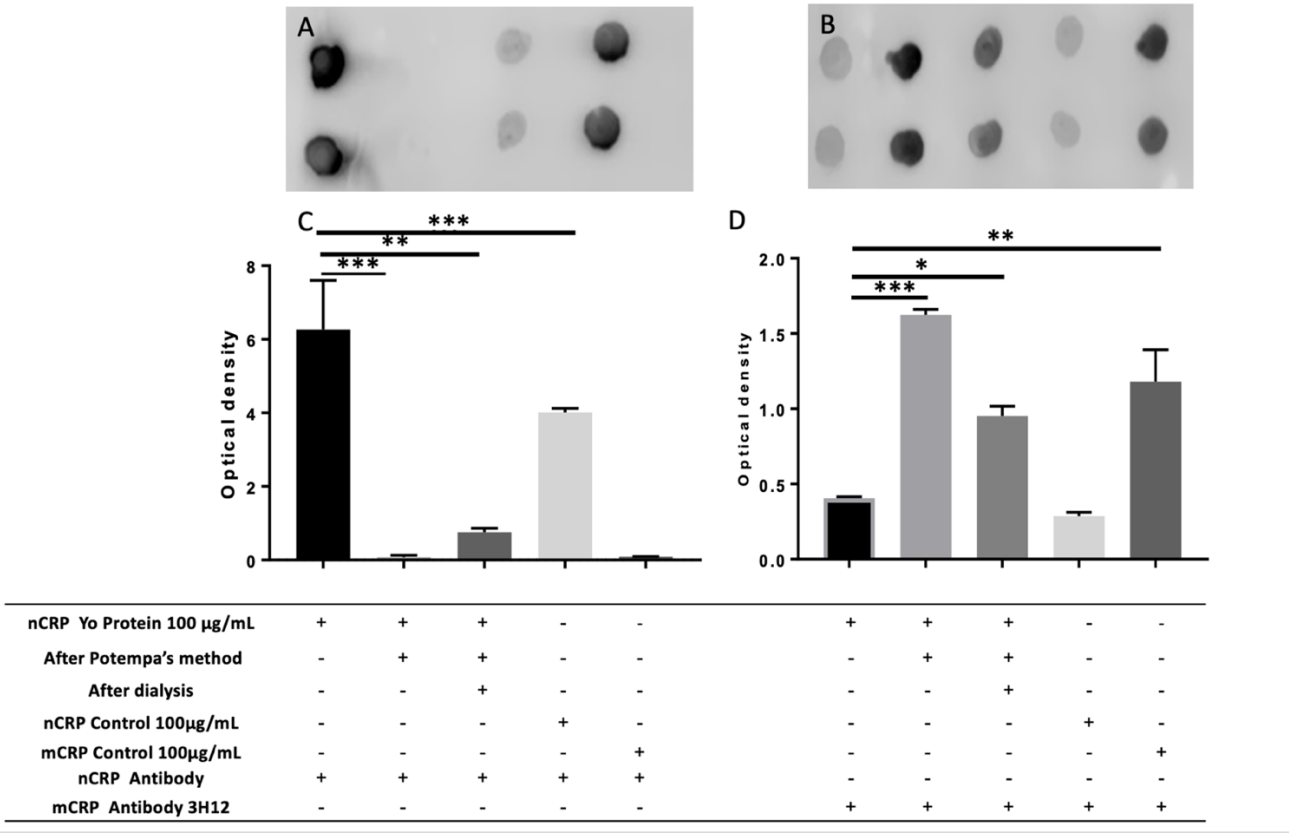


Fig. 3.2. nCRP dissociation into mCRPs. Dissociation of nCRP into its pro-inflammatory isoform mCRP was validated. A-C) After incubation and in high urea concentrations (8M) and EDTA (10mm), the anti-nCRP antibody confirmed that nCRP (100 µg/mL) showed a high dissociation from nCRP into the mCRP forms (100 µg/mL). In this dot-blot binding assay, 100 µg/mL nCRP (commercial or control) and mCRP (generated or control) were loaded on the membrane. The One-way ANOVA, together with Tukey's post-hoc, a 95% CI and a 5% α error, confirmed a statically significant difference before and after the Potempa method (p-value***<0.0005). The dialysis process reduced the mCRP concentration, partially affecting the statically significant difference obtained previously (p-value**<0.0047). B-D) After incubation and in high urea concentrations (8M) and EDTA (10mm), the anti-mCRP3H12 antibody confirmed that nCRP (100 µg/mL) had a high percentage of dissociation from the native into the monomeric forms (100 µg/mL). The One-way ANOVA, along with Tukey's post-hoc, a 95% CI and a 5% α error, confirmed a statistically significant difference before and after the Potempa method (p-value***<0.0005). The dialysis process reduced the mCRP concentration, affecting partially the statical difference obtain previously (p**<0.0091). (n=3)

Taylor and Van Den Berg (2007) reported on the possibility to dissociate nCRP into mCRP by heating the nCRP at a temperature of +70 °C for one hour. According to Taylor and Van Den Berg (2007) the protein obtained by this new method was structurally and functionally the same as that of Potempa's (Potempa et al., 1983; Potempa et al., 2015). As shown in **Fig.3.3 A,** the anti-nCRP antibody selectively bound only with the nCRP form, showing no difference between the commercial nCRP and the nCRP control.

nCRP was exposed to Potempa's dissociation method or heating **Fig. 3.3B.** Both methods dissociated the nCRP into mCRP**,** as confirmed by the anti-mCRP3H12 antibody **Fig. 3.3B.** This dissociation led to a difference between the original nCRP protein and the final product (mCRP). However, the heating process seemed to affect to mCRP yield (**Fig. 3.3B.**), which may be due to it having a higher protein degradation than the Potempa method **Fig. 3.3B.**

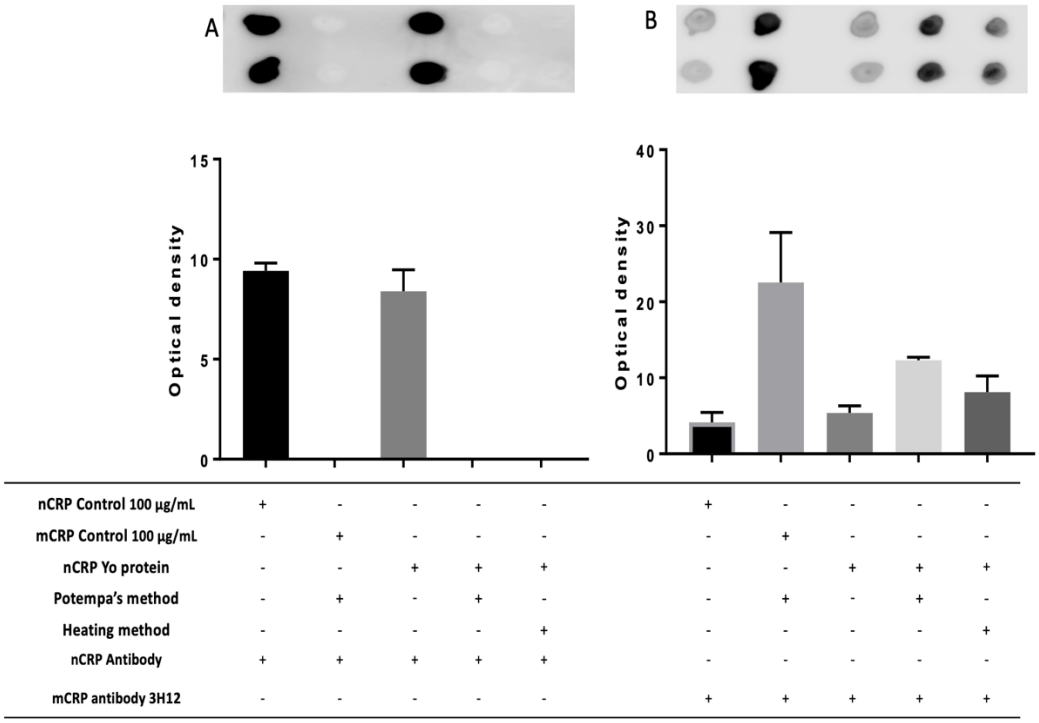


Fig. 3.3. **Dissociation methods for nCRP into mCRP.** Figure A showed no difference between the commercial nCRP and the one received from Potempa's laboratories (nCRP control). In this dot-blot binding assay, both the dissociated method (nCRP heat at a temperature of +70 °C for one hour and Potempa's procedure) were able to dissociate the nCRP into the mCRP. Antibody selectivity for the mCRP and nCRP was also assessed. The results showed that the anti-mCRP3H12 antibody was only able to bind the mCRP. This experiment was repeated once (n=1; 2 technical replicates).

3.3 SMIs binding with nCRP during and after dissociation into mCRP.

As aforementioned, both Potempa's method and heating dissociated the nCRP into the mCRP conformational form. Furthermore, the results of this study also evidenced that, when the nCRP was dissociated using Potempa's method, the anti-mCRP3H12 antibody acquired a higher binding capacity to the new conformational form than it did through heating **Fig. 3.3B.**

Whether or not the aforementioned SMIs were able to block the dissociation from nCRP into mCRP, was also evaluated. As the ACh neurotransmitter has a similar structural homology to that of the 1,6-bis (phosphocholine)-hexane (Slevin et al., 2018), a specific nCRP small-molecule inhibitor (Pepys et al., 2006), it was reasonable to target nCRP with SMIs (**Fig. 3.4**). A further two SMIs were tested for their ability to inhibit nCRP, i.e., Tacrine and Nicotine as ligands and protein-ligand computational docking was carried out to evaluate the target binding site (**Fig. 3.4**)**.** The SMIs concentration (ACh 100 μM, Tac 10 μM, or Nicotine a 150 ng/mL), were the same reported in the experiment 5.2 the SMIs were incubated in a buffer for two to four hours (the same time-point used in experiment 5.4).

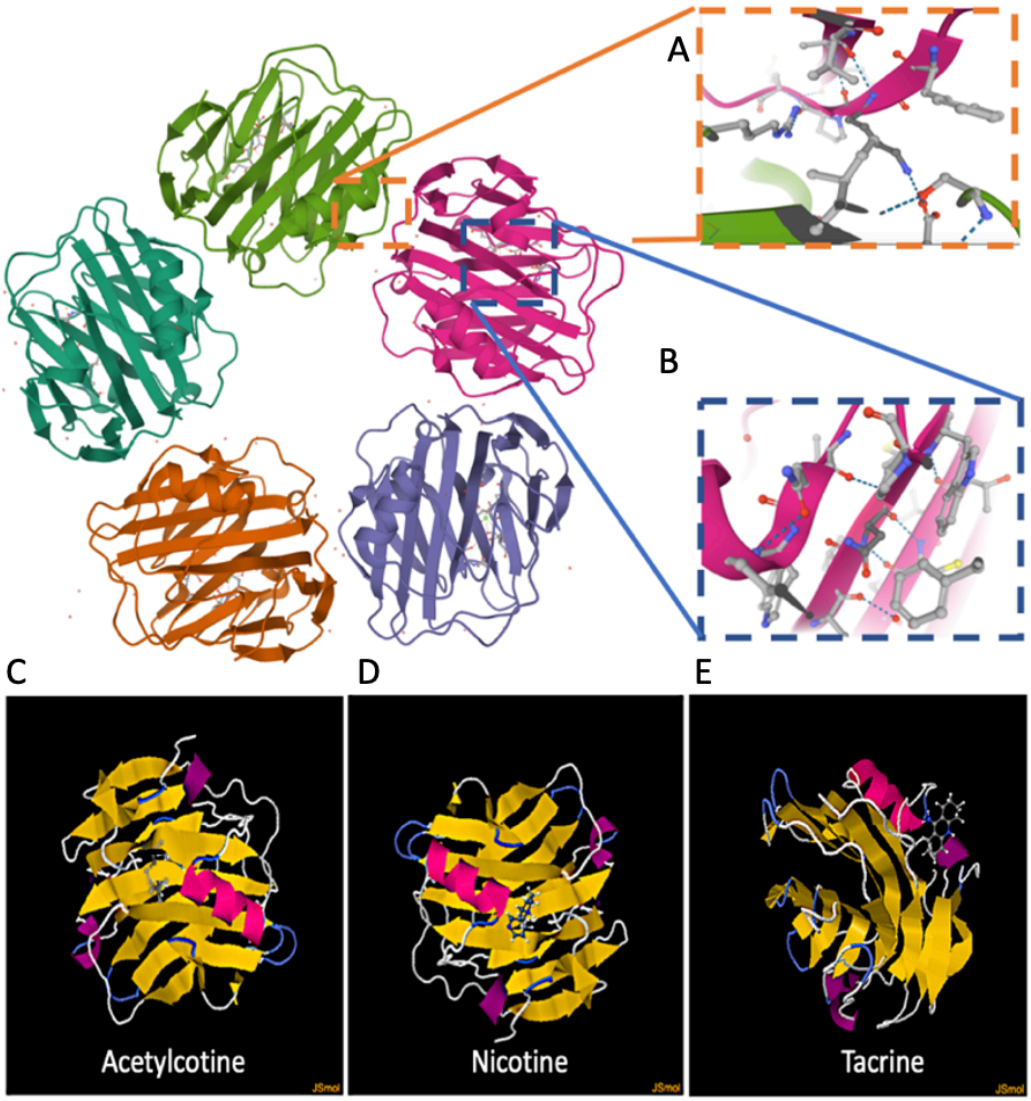
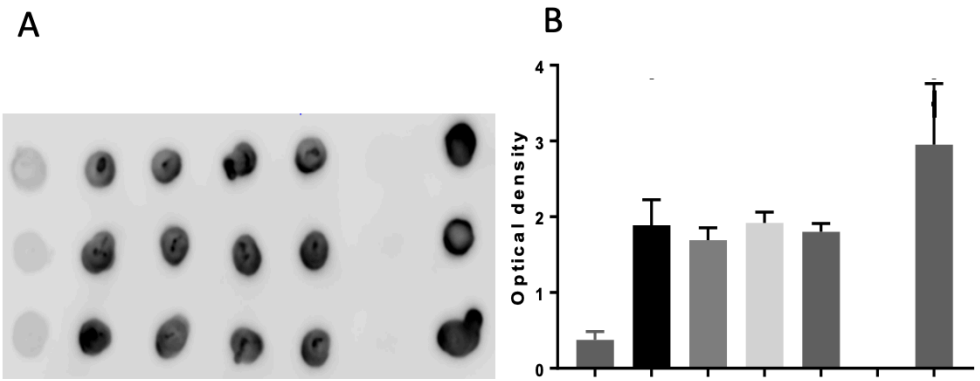


Fig 3.4. Small Molecule inhibitors (SMIs) binding site evaluation with nCRP during and after dissociation into mCRP. A) nCRP structural analysis showed two crucial sites: A) Highlighted in orange: the subunit binding site; in blue (B): the ligand binding site. Computational modelling of mCRP in combination with SMIs was carried out. The nCRP subunit docking was evaluated with C) Ach, D) Nicotine and E) Tacrine. All three SMIs bound partially with the protein but very weakly and did not bind covalently to the protein active site (Kcal/mol A:-7.29, B:-7.50, C:-7.06). Furthermore, none of the 3 SMIs occupied the aforementioned binding sites, A and B. Furthermore, none of the three SMIs did not occupy the two binding sites mentioned in A and B.

There was a difference in the binding trend for anti-mCRP3H12 antibody between the mCRP generated by the commercial nCRP+SMIs (ACh or Tacrine or Nicotine) and the control mCRP (**Fig. 3.5**). Moreover, this preliminary result also evidenced that there was no difference amongst the groups where the mCRP had been generated by the commercial nCRP, with or without the SMI pre-treatment.



nCRP Yo protein 100 µg/mL	+	-	-	-	-	-	-		+	-	-	-	-	-	-
mCRP Yo protein 100 µg/mL	-	+	+	+	+	-	-		-	+	+	+	+	-	-
ACh 100µM	-	-	+	-	-	-	-		-	-	+		-	-	-
Tac 10 µM	-	-	-	+	-	-	-		-	-	-	+	-	-	-
Nic 150 ng/mL	-	-	-	-	+	-	-		-	-	-	-	+	-	-
nCRP Control 100 µg/mL	-	-	-	-	-	+	-		-	-	-	-	-	+	-
mCRP Control 100 µg/mL	-	-	-	-	-	-	+		-	-	-	-	-	-	+
mCRP antibody 3H12	+	+	+	+	+	+	+		+	+	+	+	+	+	+

Fig. 3.5. nCRP-mCRP dissociation was not affected by SMIs. In this dot-blot binding assay, commercial nCRP (Yo protein) was used in all groups. In this dot-blot binding assays, 100 µg/mL nCRP and mCRP were loaded onto the membrane with ACh (100 µM), Tac (10 µM), or Nicotine a (150 ng/mL). The nCRP control and the mCRP received from Potempa's laboratories (mCRP control) were used as a control. The preliminary result here reported show evidenced a difference before and after Potempa's method. This provisional data also confirms that there were no differences amongst the groups where the mCRP had been generated from the nCRP from Yo protein laboratories (with or without SMI pre-treatment). (n=1-3 technical replicates).

This experiment demonstrated that SMIs are not able to block nCRP dissociation into mCRP. Furthermore, despite a similar structural homology between ACh and the 1,6-bis(phosphocholine)-hexane, this SMI was not able to prevent nCRP dissociation. The possibility of there being a potential direct bond between the pure mCRP and SMIs was investigated. To do so, the mCRP (100 µg/mL) and the SMIs were incubated in a buffer for two to four hours (the same time-point and concentration used in experiment 5.4) and no potential binding was observed between the SMIs and CRP in its monomeric form (**Fig. 3.6**).

The experiments described hereafter showed that dissociation could effectively be carried out experimentally. However, these preliminary results confirmed that the presence of SMIs did not affect the dissociation. The results herein reported suggested that SMIs do not have the capacity to block the dissociation from nCRP into mCRP nor do they directly bind with the mCRP. These preliminary results were later confirmed through surface plasmon resonance (SPR), in data published by the authors of this study (Slevin et al., 2018).

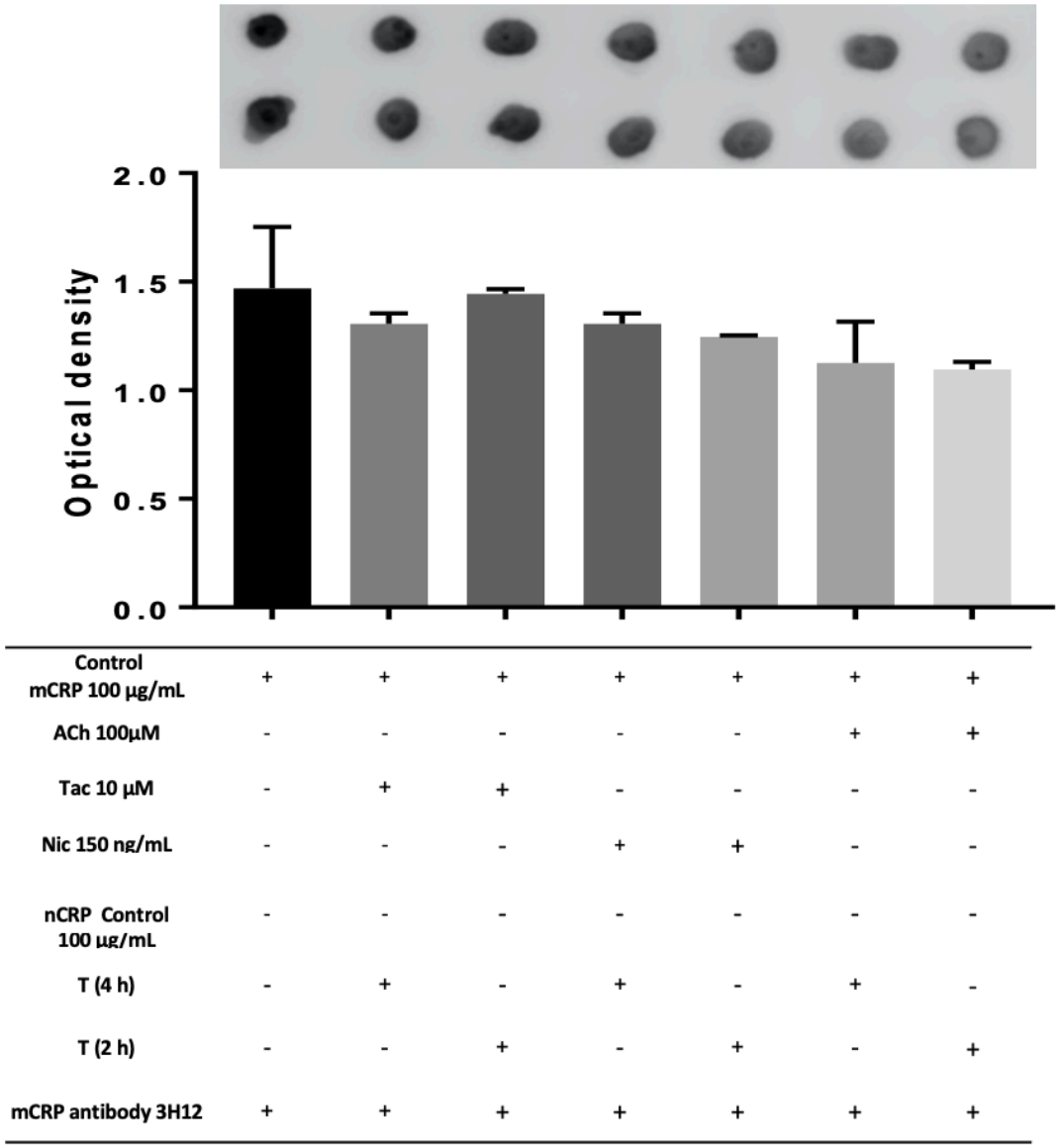


Fig.3.6. SMIs don't bind with mCRP. A dot-blot binding assay showing the anti-mCRP3H12 antibody binding affinity with the mCRP form. A total of 100 µg/mL mCRP was loaded onto the membrane with ACh (100 µM), Tacrine (10 µM) or Nicotine (150 ng/mL). The antibody maintained a high binding activity even in the presence of ACh (100 µM), Tacrine (10 µM) and Nicotine (150 ng/mL) and an equal amount of mCRP protein concentration (100 µg/mL). These preliminary results showed that SMIs do not bind with the pure mCRP form. (n=1-2 technical replicates).

3.4 Discussion.

A perpetual low-chronic inflammatory (LCI) state is correlated with an increase in nCRP levels (Dinh et al., 2019) and the probability that atherosclerosis will be promoted (Ambrose and Barua, 2004; Libby, 2012; Siasos et al., 2014; Poulsen et al., 2015; Katakami, 2018). LCI leads to endothelial cell (EC) damage (Parnell et al., 2012), increases the presence of signs of injury and impairs tight junctions (Pant et al., 2014). Activated platelets are involved in atherogenesis progression (Gawaz et al., 2005; Nording et al., 2015) dissociating the nCRP into mCRP and its accumulation in the atherosclerotic necrotic core, further increasing the local pro-inflammatory activity (Eisenhardt et al., 2009b). For this reason, this research was to establish whether SMIs, i.e., ACh, Tacrine and Nicotine, have the ability to block or at least impair nCRP-mCRP dissociation in order to moderate the level of inflammation caused by the mCRP.

The first step was to determine the ability antibodies have (anti-nCRP and anti-mCRP3H12) to selectively bind with their respective protein target. A second experiment was carried out to confirm the dissociation of nCRP into the mCRP form. This was followed by a comparison between the two types of dissociation methods. The last experiments assessed whether the previously selected SMIs were able to block, or at least reduce, this dissociation.

The antibodies were able to specifically bind with their respective proteins. As previously described by Potempa et al. (1983) and Potempa et al. (2015), urea (8 M) and EDTA (10 mM) led to the dissociation of nCRP into mCRP (**Fig. 3.2**). It was also observed (**Fig. 3.3**) that the nCRP dissociation occurred when the nCRP was heated at +70 °C for one hour (Taylor and Van Den Berg, 2007). However, this method also influenced the capacity the anti-mCRP3H12 antibody had to bind with the insoluble pro-inflammatory form (mCRP) and had a lower efficacy.

Next, the SMIs were used to prevent nCRP dissociation. However, although ACh has some structural similarity to1,6-bisPC, it was unable to block

dissociation (**Fig. 3.5**) nor did it interact with the mCRP alone (**Fig. 3.6**), in line with the surface plasmon resonance (SPR) reported in a recent publication from this work (Slevin et al., 2018). This was also observed when the two other SMIs were tested, i.e. neither Nicotine nor Tacrine were able to prevent dissociation.

The fact that the SMIs did not prevent nCRP dissociation might be due to the different binding site locations between the SMIs and the ruptured nCRP sites. The computational docking analysis showed that all SMIs bound on the edge of the nCRP subunit, which might not suffice to avoid its dissociation. Furthermore, maybe the fact that the SMI ACh, despite their similarity to 1,6-bisPC, do not bind to nCRP or bind very weakly, could explain the feeble charge. However, an interesting finding came to light i.e. the observation of antibody selectivity, which could be used as a strategy target mCRP and mitigate its inflammatory effect downstream.

3.5 Key findings:

The key findings of the experiments described in this chapter are:

- The selective identification of the two different forms of CRP (nCRP and mCRP), via antibody strategy, was investigated by dot-blot binding assays this evidenced: *(I)* that there are two different conformational forms; *(II)* that the antibodies used were able to bind separately both with the native and the monomeric CRP forms. Both the anti-nCRP and anti-mCRP3H12 antibodies had the same binding selectivity capacity with the appropriate conformational form.

- The second step confirmed that both dissociative methods tested, i.e., Potempa's and heating, were able to dissociate the nCRP into mCRP and to evaluate how the ratio nCRP/mCRP is affected during the dissociation step. Moreover, dot-blot binding assay confirmed dissociation from nCRP into the mCRP by both methods (Potempa's and heating), however, both methods affected how the anti-mCRP

antibody (anti-mCRP3H12) bound to monomeric CRP.

- One of the goals was to determine whether selected SMIs had the ability to block the dissociation of nCRP-mCRP. Even in the presence of ACh, Tacrine and Nicotine and an equal amount of protein concentration (see paragraph 3.3 for concentrations) the antibody maintained a high binding activity. This result, along with those obtained by the SPR evidenced that SMIs did not affect dissociation, nor did they have any binding activity with the pure mCRP form.

3.6 Summary.

A high nCRP (>2.2 mg/L) blood level has been associated with increased inflammation. Moreover, nCRP has been associated with a wide variety of conditions mostly related to inflammation pathways, these range from infection to cancer and a high risk of heart attack (Sproston and Ashworth, 2018). Despite this, the correlation between these pathologies and nCRP/mCRP remains elusive. In an effort to shed some light on the underlying mechanisms that lead to the dissociation of the nCRP into mCRP, the selectivity and specificity the antibody has, as well as the role SMIs play in reducing the dissociation from nCRP to mCRP, was evaluated.

The aforementioned experiments demonstrated that dissociation could be successfully carried out experimentally. Even if this data showed that the SMIs were unable to block the dissociation from nCRP into mCRP, the antibody selectivity between the two forms nCRP and mCRP provided promising results as to the possibility of using therapeutic antibody strategies to mitigate the level of inflammation triggered by the effect of mCRP. However, future studies are necessary to validate the use of antibody therapy against mCRP in humans.

CHAPTER 4 RESULTS: Assessment of whether mCRP stimulation induces monocyte -derived macrophage pro-inflammatory activity.

CHAPTER 4: Assessment of whether mCRP stimulation induces monocyte-derived macrophage pro-inflammatory activity.

Atherosclerotic disease (ADs) is a pathological physiological condition affecting the cardiovascular system which increases the concentration of lipoprotein(a), plasminogen activator inhibitor-1[41], nCRP and monocytes count (Arenillas et al., 2008; Flynn et al., 2019). It has been observed that ADs platelet activation dissociates nCRP into mCRP, which accumulates in the aorta, promoting the development of carotid artery atherosclerotic plaques (Eisenhardt et al., 2009b) which are a known risk factor for Myocardial Infarction and (MI) ischemic stroke (IS) (Swirski and Nahrendorf, 2013; Yaghi et al., 2019).

The first medical intervention for both MI and IS should be targeted to restoring the blood flow (Pisters and Lip, 2013; Howard, 2016). However, once this goal has been accomplished, the ischemia/reperfusion injury (IRI) facilitates the accumulation of both native C-reactive protein (nCRP) and monomeric CRP (mCRP) in re-perfused tissue (McFadyen et al., 2018). Indeed, two studies have already demonstrated the accumulation of nCRP and mCRP in post-ischemic cerebrals tissues (Slevin et al., 2010; Napoli et al., 2018). When mCRP is accumulated in the brain it increases the pro-angiogenic effects induced by the activation of the extracellular signal-regulated kinase (ERK) (Turu et al., 2008) and the PI3K/Akt survival intrinsic pathway (Boras et al., 2014) in endothelial cells (ECs). Eisenhardt et al. (2009b) reported that the mCRP pro-angiogenic and pro-inflammatory effects in ECs are mediated by FC-γ receptors (FCγRs). Thiele et al. (2014a) confirm that these pro-angiogenic and pro-inflammatory effects are associated with an increased expression of $CD68^{+}$ monocytes and reactive oxygen species (ROS) production in proximity to post-ischemic tissue. This provides a possible explanation as to why monocyte accumulation in post-ischemic tissue has been observed in both IS and MI (Chiba and Umegaki, 2013; Swirski and Nahrendorf, 2013).

As aforementioned in chapter 1, paragraph 1.2.2.1 after recruitment from the systemic circulation, monocytes differentiate into naïve M0 resting macrophages at the site of injury (Malissen et al., 2014; Orekhov et al., 2019). After this initial resting state, macrophages polarize into pro (M1) or anti (M2) inflammatory phenotypes depending on the stimuli that they have received at the injury site (Chistiakov et al., 2015). That is, this polarization seems to depend on the presence of both Pathogen Associated Molecular Patterns (PAMP) and Damage-associated molecular patterns (DAMPs) released due to cellular stress or tissue injury (Liu et al., 2017a). When polarization takes place, these cells express new surface makers, e.g., F4/80, CD11b, CD18, CD68, colony stimulating factor 1 receptor (CSF1R) and increase the human FCγRs (van de Winkel and Anderson, 1991; Rothe et al., 1996; Murray and Wynn, 2011).

FCγRs are expressed on cells of the immune system, such us monocytes and macrophages, (van de Winkel and Anderson, 1991; Rothe et al., 1996) and are also considered the principal nCRP-binding receptors (Tron et al., 2008). Literature reports that when nCRP binds with these receptors, the immune system releases both pro and anti-inflammatory cytokines (Ferrarese et al., 1999; Lu et al., 2018). However, whilst some authors suggested that this binding is a fundamental step involved in inflammatory activity (Lu et al., 2012), others researcher evidenced that identifying which form of CRP (nCRP or mCRP) has a pro or anti-inflammatory effect is challenging (Tanigaki et al., 2015). Later, these doubts were clarified by other studies which reported that while mCRP is able to increase the release of Nitric Oxide (NO) in monocytes, they also confirmed that nCRP reduces the quantity of NO released in the same type of cells (Sproston et al., 2018). Furthermore, other studies reported that if macrophages are stimulated with mCRP they polarize into an M1 pro-inflammatory phenotype (Trial et al., 2016), whilst if they are stimulated with nCRP, macrophages release a high amount of M2 IL-10 anti-inflammatory cytokine (Pilling et al., 2017; Moore et al., 2001). However, at the best of our knowledge currently there no data available that clarify whether mCRP is capable of promoting the release of pro-inflammatory cytokines in U937 monocyte-derived macrophages.

Therefore, knowing that monocyte infiltration and mCRP accumulation in ischemic tissue is a typical consequence of ischemic events (Slevin et al., 2010; Gronberg et al., 2013; Swirski and Nahrendorf, 2013; McFadyen et al., 2018), raises the hypothesis that the mCRP may also bind with the newly recruited monocyte-derived macrophages through the FCγRs, triggering the downstream inflammation cascade. So as to enhance the know-how as to the role mCRP plays in inflammation, the possibility that mCRP binds with monocyte-U937 derived macrophages was investigated. Furthermore, it was also tested whether mCRP pro-inflammatory features could potentially be mediated by FCγRs.

Aim and objectives: To establish whether and how mCRP has a pro-inflammatory effect in monocyte- derived macrophages.

This was addressed by the following objectives:

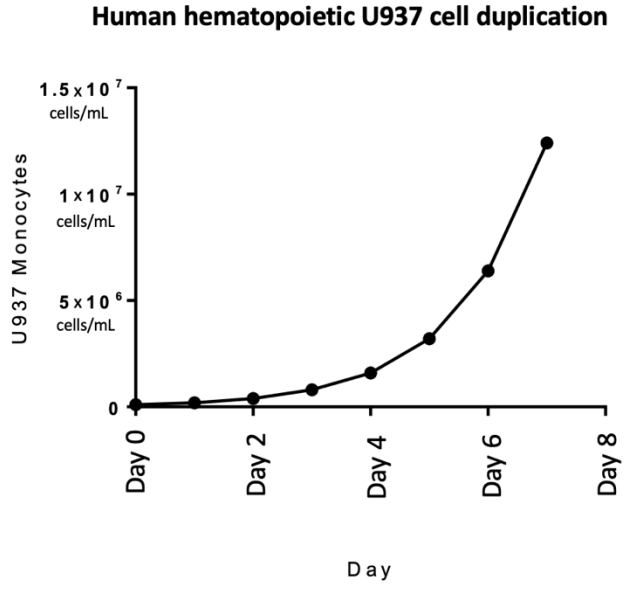
- Literature reports that both monocytes and macrophages play a pivotal role during and after an inflammatory response (Atri et al., 2018). This fundamental role has been associated with their capacity to release both pro and anti-inflammatory cytokines (W. Xu et al., 2013). This has led to a series of experiments, carried out to clarify whether the U937 monocytes and macrophages were able to release pro or anti-inflammatory cytokines.

- Literature confirms that mCRP is capable of polarizing macrophages into a specific M1 pro-inflammatory phenotype (Trial et al., 2016). Subsequently, it has been reported that, when mCRP binds monocytes chemotaxis activity is enhanced as is the concentration of NO and iNOS (Fujita et al., 2014; Sproston et al., 2018). However, none of these studies reported whether mCRP is able to control the pro and anti-inflammatory cytokine release following monocyte/macrophage cell interaction. Thus, the mCRP interaction with monocyte and macrophage cells and its effect on the cytokine cascade was evaluated.

- FCγRs are specific markers able to discriminate the monocyte-derived macrophages from dendritic cells (DCs) (Satpathy et al., 2012; Bain and Mowat, 2014). Thiele et al. (2014a) reported that mCRP triggered monocyte pro-inflammatory activity when bond to FCγRI (also known as CD64) and/or as FcγRIII (also known as CD16) receptors. However, similar study on macrophages has not been covered yet, and its inflammatory role has to be examined. It was also evaluated whether mCRP was capable of inducing inflammation via binding to FCγRs and whether anti-FCγRs antibodies inhibit the binding of mCRP and macrophages, reducing the mCRP pro-inflammatory activity.

4.1 Human haematopoietic U937 cell duplication.

Preliminary data on U937 replication time frame evaluation was carried out to optimize the experiments performed during this study. As shown in **Fig. 4.1** they duplicated each day. As shown in the graph **Fig. 4.1** and **Table 4.1,** enough cells to perform the experiments were obtained (1.24 x10^7 cells) in one week.



Date	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
N° cells/mL Average of 3 different T-75 Cell Culture Flask	100000	198000	399000	810000	1590000	3200000	6389000	12400000
% Cell Duplication	100 %	198 %	399 %	810 %	1590 %	3200 %	6389 %	12400 %

Fig 4.1 and **Table 4.1**. **Human haematopoietic U937 cell duplication.** Cell replication of the U937 monocyte cell line in a growing media (RPMI 1640 with L-glutamine Lonza with 10% FBS F9665 Sigma®) was tested from passage 10 to 14 in a T-75 cell culture flask. Cell counting was done by the BioRad TC10™ automated cell counter. The U937monocyte cell sub-culturing was loaded onto a dual-chamber slide (BioRad Cell Counting Slides for TC10™ dual chamber), with 10 µL of sample solution (containing 5 µL of cell suspension and 5 µL of trypan blue dye). The slide was then put into the TC10™ automated cell counter to obtain the total cell count, the percentage of cell viability and the number of live cells (n=3).

4.2 U937 monocyte stimulation with Lipopolysaccharides (LPS) and mCRP assessment of pro-inflammatory effects.

After evaluating the U937monocyte duplication rate, this cell line was used to assess their potential role in triggering the inflammation process. As the LPS found in the outer membrane of Gram-negative bacteria are capable of inducing an acute inflammatory response on mononuclear cells (PBMCs) and monocytes (Ngkelo et al., 2012) it was used as a positive control. TNF-α was selected as a reference, as previously reported (van der Bruggen et al., 1999) to assess whether monocytes are able to release pro-inflammatory cytokines. mCRP concentration (100 μg/mL) and time-point (24 hours) were the same as those used in experiment 4.5, where the mCRP biological activity on monocyte-derived macrophages was tested.

Although the monocytes were exposed to LPS (100 ng/mL) they did not release TNF-α pro-inflammatory cytokines. This datum is in line with previously published experiments which confirmed that a pro-inflammatory response could be initiated only after differentiation from monocytes to macrophages (Hida et al., 2000).

As shown in **Fig. 4.2,** enzyme-linked immunosorbent assay (ELISA) was carried out on the monocyte media that had been stimulated by LPS (100 ng/mL) or mCRP (at 100 μg/mL). There was no statistical significance in any of the conditions.

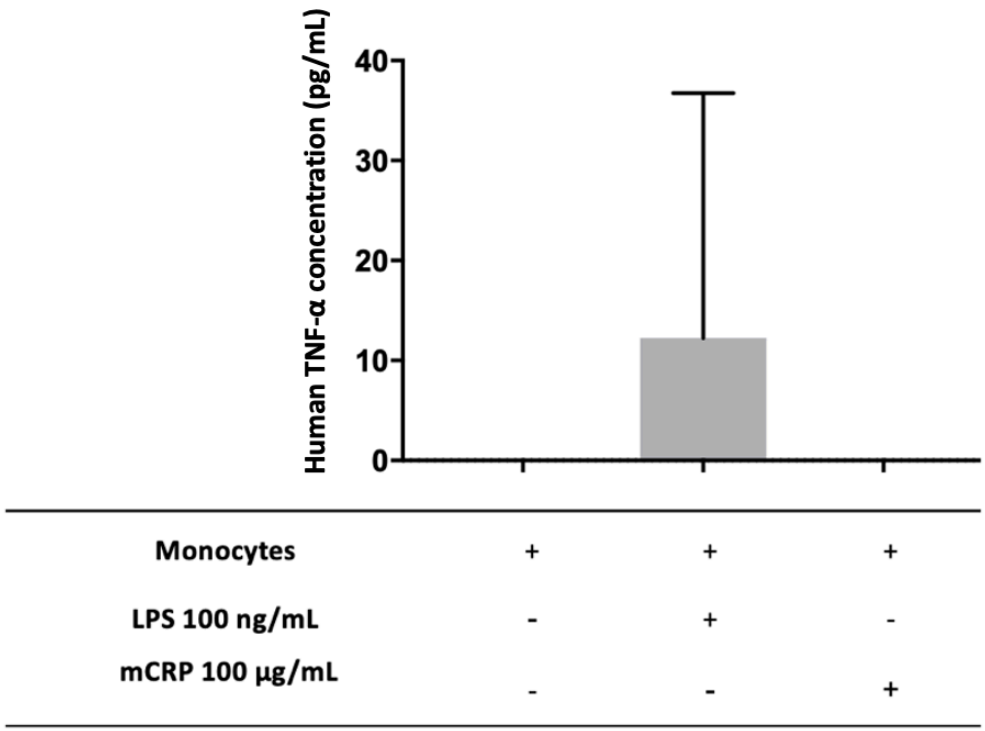


Fig.4.2. Monocytes did not release TNF-α pro-inflammatory cytokines. The U937 monocytes were exposed to LPS (100 ng/mL used as the positive control) or to mCRP (100 µg/mL) for 24 hours in a growing media (RPMI 1640 with L-glutamine Lonza with 10% FBS F9665 Sigma®). TNF-α production in the supernatant was measured by ELISA kits (R&D System). The results are shown as the mean ± SD. The One-way analysis of variance (ANOVA) and Tukey's post-hoc test with a CI 95% and a fixed 5% α error confirmed that there were no significant differences amongst the groups. The U937 monocytes did not release any significant TNF-α (<20 pg/mL), even in the presence of LPS at a concentration of 100 ng/mL). (n=3)

4.3 Phorbol-12-myristate 13-acetate (PMA) as a treatment: for cell differentiation and viability.

After ascertaining that U937 monocyte cell lines were unable to release significant concentrations of TNF-α, in line with a previous study (Hida et al., 2000), the development of an inflammation *in vitro* model was the next step.

Firstly, monocyte differentiation in macrophages was assessed. As aforementioned in chapter 1, paragraph 1.2.2.2, macrophages are principally divided into the M1 (pro-inflammatory) and M2 (anti-inflammatory) subtype and have different biological and functional proprieties (Ley, 2017). However, before reaching these final states, monocytes differentiate into the macrophage resting subtype (M0) (Zhao et al., 2017). Previous studies have reported that PMA is able to differentiate the U937 monocytes into macrophages (Passmore et al., 2001) in the resting M0 subtype without affecting their polarization (Song et al., 2015). The macrophages were then differentiated using the U937 differentiation method (PMA at concentration of 50 ng/mL for 72 hours), according to the Sproston et al. (2018) study. This experiment was carried to observe how what effect PMA had on U937 cell viability (**Table 4.2** and **Fig 4.3**) and differentiation (**Fig 4.4** and **Fig.4.5**).

As shown in **Fig. 4.3**, after PMA treatment for 72-hours (50 ng/mL), it was observed that cell viability had been significantly affected. Only 10% of the initial concentration (2 x 10^6 cell/mL) attached to the 6-well plate (2 x 10^5 cell/mL).

	Monocytes	Macrophages (after PMA treatment)
1 well	2000000	177000
2 well	2000000	199000
3 well	2000000	167000
Avarage	2000000	181000
% Cell Viability	100 %	9.05 %

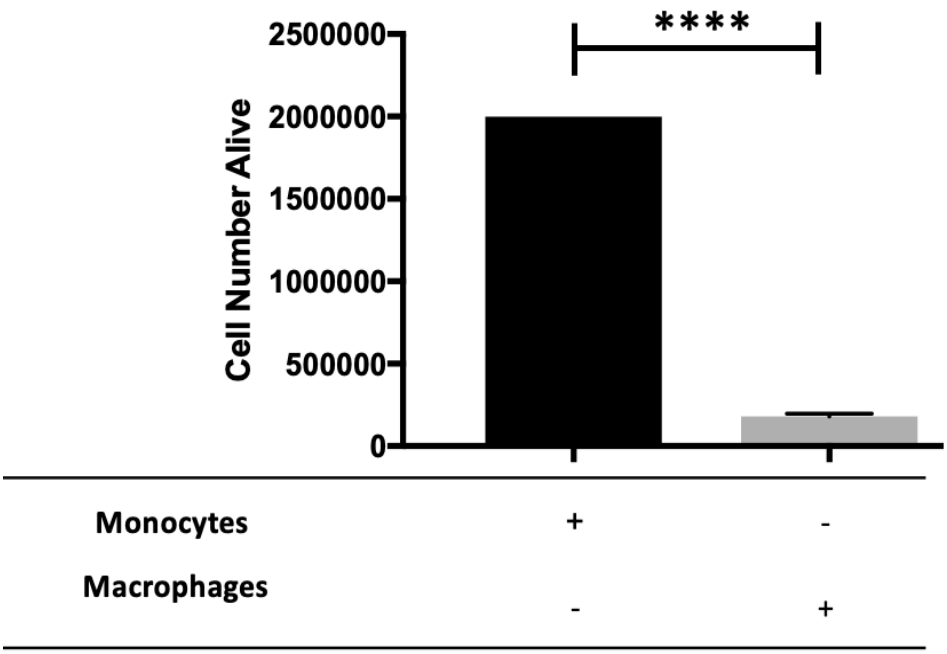


Table 4.2. and **Fig. 4.3**. **Phorbol-12-myristate 13-acetate (PMA) as a treatment: for cell differentiation.** Differentiation from floating monocytes into adherent macrophages was induced by exposing the U937 cells to a differentiated media containing RPMI 1640 with L-glutamine (1640 medium Lonza) supplemented with 10% of FBS (F9665 Sigma®) and phorbol-12-myristate 13-acetate (PMA793461 Sigma®) at 50 ng/mL for 72 hours. After PMA treatment, 20 µL of unattached or attached cells were placed into an eppendorf tube containing 20 µL of trypan blue solution. This was then loaded onto a dual-chamber slide. The slide was then put into the TC10™ automated cell counter to obtain the total cell count, the percentage of cell viability and the number of live cells. The results are shown as the mean ± SD. ****P < 0.0001. As can be observed in the figure, the U937 cell viability was significantly reduced by PMA. The one-tailed unpaired t-test with a 99% CI and a 1% α error confirmed the substantial reduction of cell viability before ($2x10^6$ cells/well) and after ($2x10^5$ cells/well) PMA use (p-value<0.0001). Table 4.2 shows how only 9.05 % of U937 cells were viable after exposure to PMA. (n=3)

After having ascertained whether cell viability was affected by PMA treatment, the differentiation of monocytes into macrophages after PMA treatment was evaluated. At this stage, the unattached cells were removed and only untreated and treated (macrophages) were tested by the FACS analysis.

Fig. 4.4 and **Fig 4.5** depict the differentiation from monocytes into macrophages. **Fig. 4.4A** shows monocytes as suspension cells that are clearly separated one from the other. This fundamental characteristic is lost after PMA treatment. Following differentiation **Fig. 4.4B**, where macrophages are also able to form clusters**,** they go through morphological and phenotypic changes, allowing them to attach to the plastic flask.

The next step, after 72 hours of PMA stimulation, was FACS analysis using the anti-huCD11c antibody (Wentworth et al., 2010; Yu et al., 2016; Sproston et al., 2018). As shown in **Fig**.**4.5**, PMA-differentiated cells substantially increased the CD11c macrophage marker (40.8% CD11c+), whilst monocytes lacked the CD11c macrophage marker (0.0% CD11c+). **Fig. 4.5** shows a significant difference (p-value<0.0001) in Human-CD11c expression before and after PMA treatment and between the groups (undifferentiated U937 monocytes vs differentiated U937 monocytes), confirming macrophage differentiation.

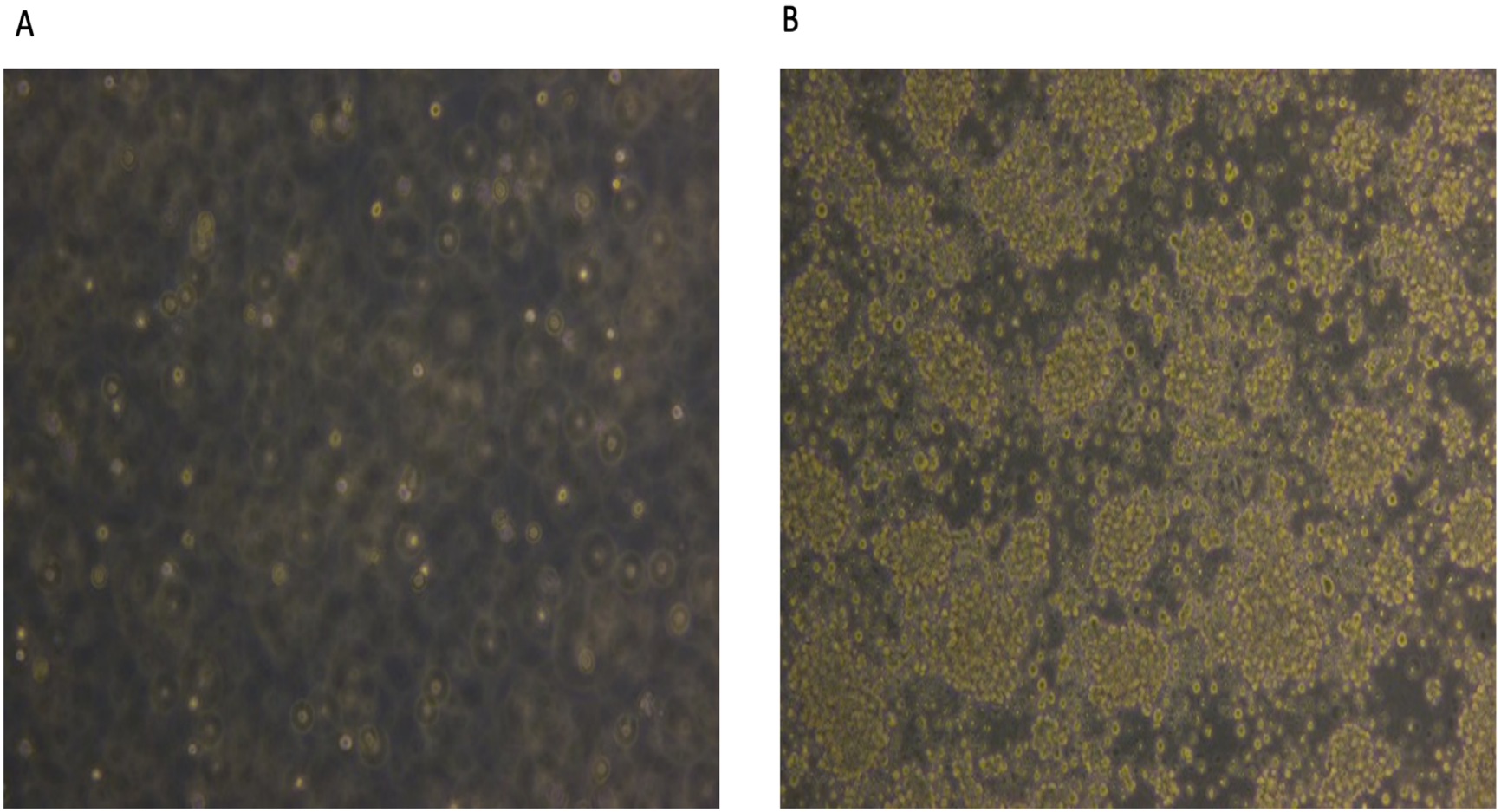


Fig. 4.4. Monocytes and macrophages. Fig (A) U937monocyte cells were grown in a growth medium (RPMI 1640 with L-glutamine Lonza) with 10% FBS (FBS F9665 Sigma®) in humidified air at 95% with 5% CO_2 at 37°C in a T-75 flask from passage 10 to 14. Following PMA treatment (50 ng/mL for 72-hours), suspension cells (monocytes) became monocyte-derived macrophages and attached to the well (B). Following PMA treatment, macrophages formed clusters (B).

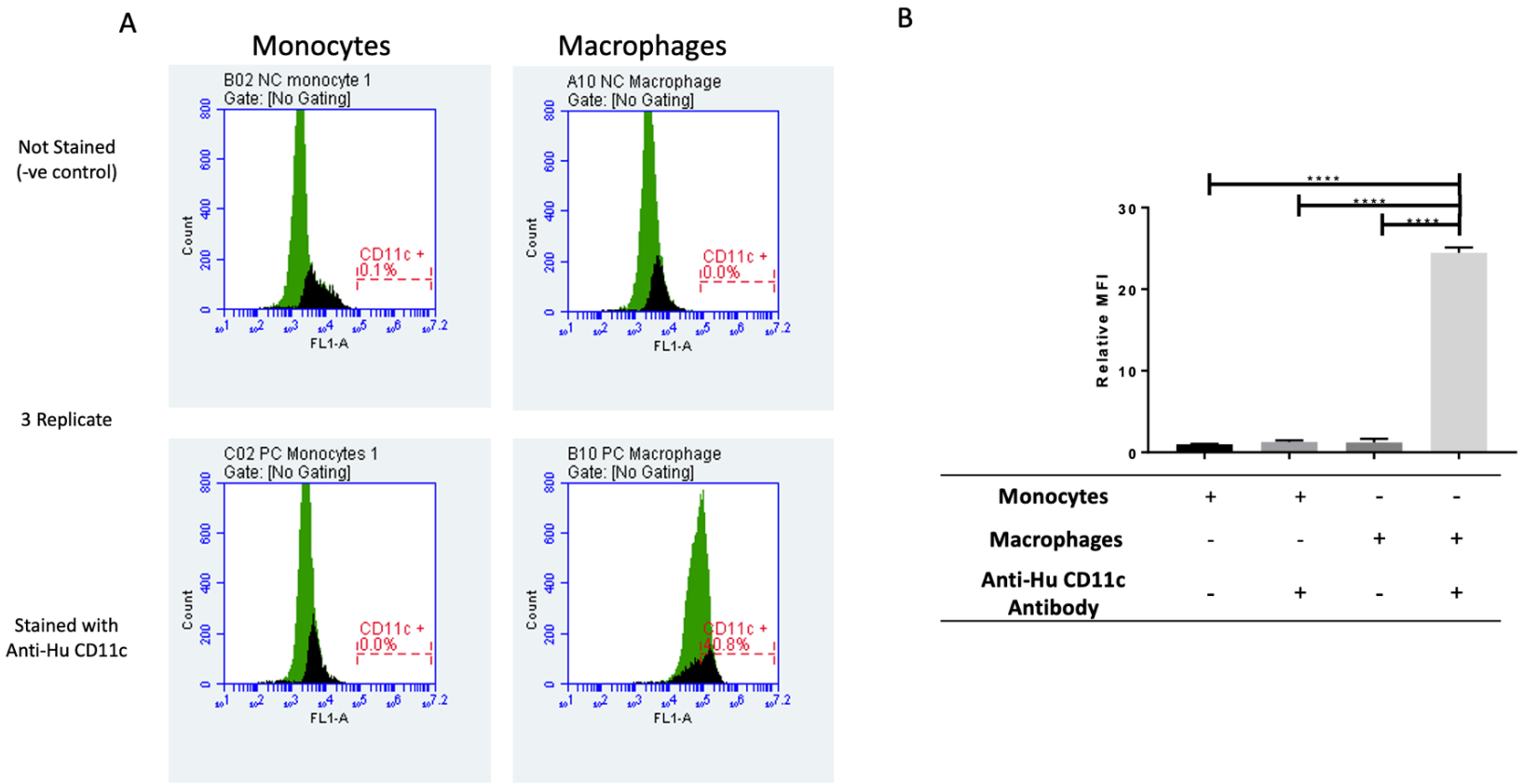


Fig 4.5. Macrophages PMA-differentiated significantly increase CD11c macrophage marker. After 72 hours of PMA stimulation U937monocyte cells cultured into RPMI 1640 with L-glutamine (1640 medium Lonza) with 10% of FBS (F9665 Sigma®) and PMA at 50 ng/mL for 72 hours, were examined by FACS analysis by using anti-huCD11c (Thermo-fisher Anti–Hu CD11c LOT2008210) in order to confirm monocytes vs macrophages differentiation. Results showed a significant difference as the mean ± SD. ****p-value < 0.0001. Fig.4.5A. Two divergent populations of cells were identified. One-tailed unpaired t-test with a 99% CI and a 1% α confirm a significant difference (p-value< 0.0001) before and after PMA treatment. CD11c macrophages markers (40.8% CD11c+) is not present monocyte (0.0% CD11c+). (n=3).

4.4 U937 macrophages model to study inflammation.

The ELISA test was performed after FACS to assess the functional differentiation for U937 monocytes into U937 macrophages. On the basis of findings published in literature, it was presumed that PMA stimulation differentiates monocytes into macrophages (Sproston et al., 2018). When this differentiation happen, macrophages are then able to increase the cytokine production (Hida et al., 2000). This assay evaluated the ability the U937 monocyte-derived macrophages had to release the TNF-α, IL-6 and IL-10 cytokine. The macrophages were stimulated with LPS, as it has the capacity to stimulate macrophages to release both pro and anti-inflammatory cytokines, such us TNF-α, IL-6 and IL-10 (Tucureanu et al., 2018). TNF-α was tested as it is has been reported that this pro-inflammatory cytokine plays an essential role in numerous inflammatory diseases and is predominantly released by macrophages (Parameswaran and Patial, 2010). IL-6 was selected for its relevant role in several situations relating chronic inflammation (Barnes et al., 2011). IL-10, which has anti-inflammatory effects, was assessed as it as it is released by M2 (Shi et al., 2019).

The capacity macrophages have to release high amounts of pro-inflammatory cytokines, as shown in **Fig 4.6,** was confirmed through the TNF-α and it was observed that only the PMA treated cells (monocyte-derived macrophages) had the ability to release the TNF-α cytokine (p-value<0.0001).

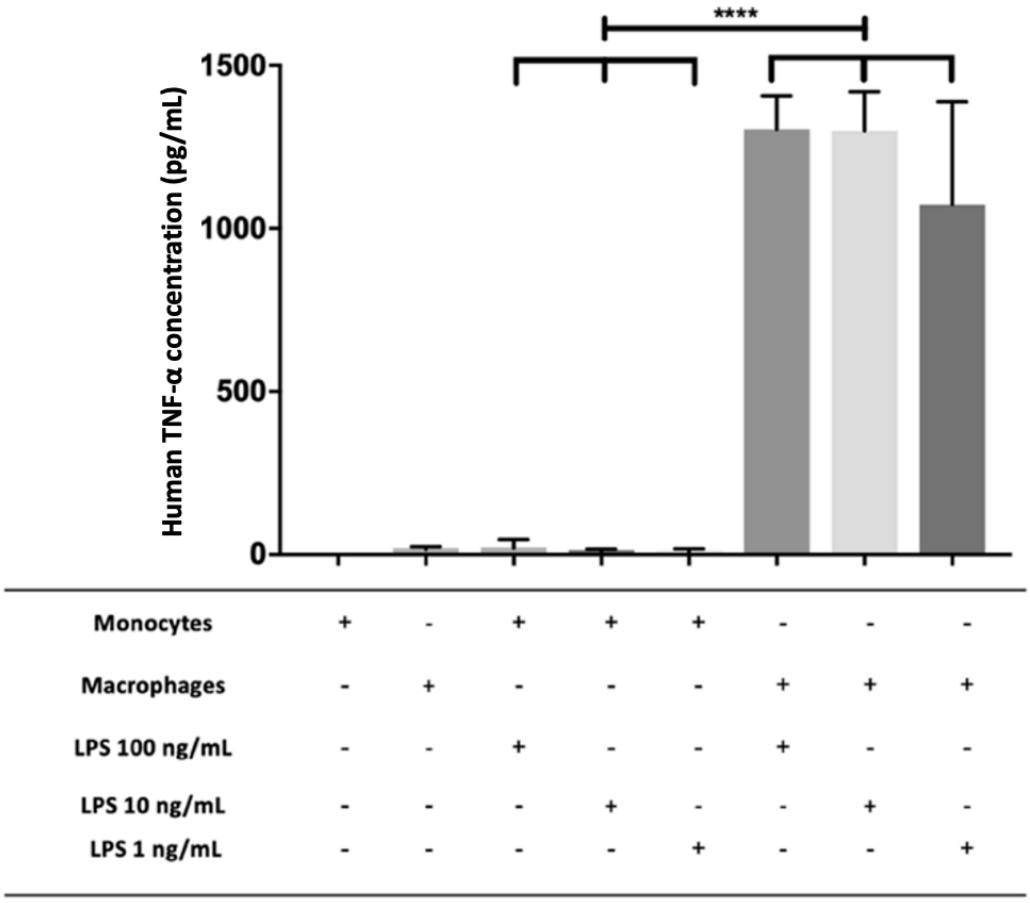


Fig. 4.6. TNF-α pro-inflammatory cytokine is released from macrophages. After a 72-hour PMA treatment (50 ng/mL), the monocyte-derived macrophages were stimulated with LPS at a concentration of 100, 10 and 1 ng/mL for 24 hours. The TNF-α production was quantified by ELISA kits (R&D System). The results are shown as the mean ± SD. ****p-value < 0.0001. As shown in figure, after PMA differentiation the macrophages were capable of releasing the TNF-α pro-inflammatory cytokine when stimulated by LPS. The One-way ANOVA test, followed by the Tukey Post-doc test with a 99.9% CI and a α error fixed at 0.01%, confirmed a statistical difference (p-value <0.0001) between monocyte groups and macrophage groups. (n=3).

The results also evidenced that the release of TNF-α pro-inflammatory cytokine was consistent and independent from the LPS concentration. Moreover, they indicated that macrophages can provide a useful pathobiological model to study inflammation.

Further experiments were set-up to optimise LPS as a positive control to evaluate other cytokine levels. Based on the confirmation that TNF-α pro-inflammatory cytokine was consistent and independent from LPS concentration (**Fig 4.6**), LPS at 10 ng/mL was selected as a positive control to assess whether macrophages are capable of increasing the IL-6 and IL-10 cytokine concentrations.

As reported in **Fig. 4.7,** the ELISA test showed that LPS stimulation (10 ng/mL) led to the production of macrophage-derived IL-6 and IL-10. A statistical difference (p-value<0.0001) in the IL-6 concentration in the presence or absence of LPS (10 ng/mL) was assessed at three set time points, i.e., 6, 12 and 24 hours.

A similar result was reported for IL-10 (**Fig. 4.7**)**.** There was a statistically significant increase in macrophage IL-10 production after LPS stimulation at 10 ng/mL. However, differently to IL-6, the IL-10 release did not remain constant over the three time points, reaching its maximum level at 12 hours.

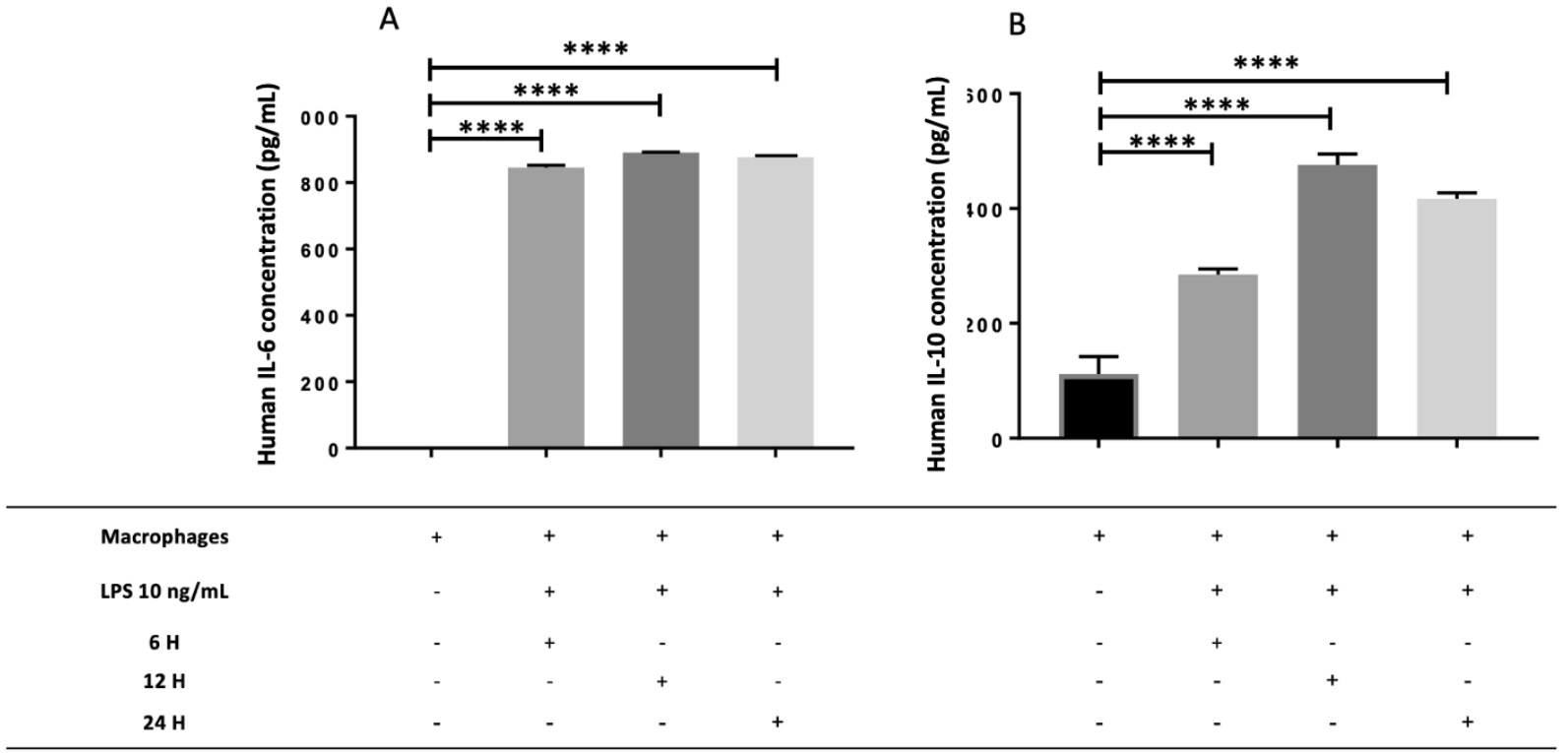


Fig 4.7. Macrophages are responsible for releasing IL-6 and IL-10 protein cytokines. After 72-hour PMA exposure (50 ng/mL), the macrophages were stimulated with LPS at a concentration of 10 ng/mL for 6, 12 and 24 hours. The IL-6 (A) and IL-10 cytokine release was quantified by ELISA kits (R&D System). The results are shown as the mean ± SD. ****P-value < 0.0001. As shown in figure, after PMA stimulation, the macrophages were able to release both the IL-6 and the IL-10 cytokine when stimulated with LPS. The One-way ANOVA test, with a 99% CI and a 1% α error, showed a statistical difference (p-value<0.0001) between the unstimulated and the LPS stimulated macrophages groups (A). A statistical difference (p-value<0.0031) was observed in the IL-10 concentration between the unstimulated macrophage group and the group where the macrophages were stimulated by LPS for 6 hours (IL-10 release: 111.5 vs 284.5 pg/mL, respectively). This difference was also confirmed in the group stimulated for 12 (111.5 vs 475.9 pg/mL, respectively) and 24 hours (111.5 vs 416.7 pg/mL respectively) (p-value<0.0003). A statistical difference (p-value=0.0021) in IL-10 concentration between the 6 and 12 hours LPS groups (284.5 vs 475.9 pg/mL respectively) and the 6 and 24 hours LPS groups (284.5 vs 416.7 pg/mL respectively) was confirmed (p-value=0.0085). No significant difference was observed between the group that had been stimulated for 12 hours and the one stimulated for 24 hours (475.9 vs 416.7 pg/mL respectively). (n=3).

4.5 Monomeric-CRP increased the IL-1β gene expression.

After evaluating the macrophage response, in terms of cytokines after LPS stimulation, the effect of the monomeric C-reactive protein and its contribution on pro-inflammatory effects were observed. mCRP is mainly characterized as a pro-inflammatory protein in different cell types, such as ECs, leukocytes and platelets (Thiele et al., 2014a; Wu et al., 2015). The effect of mCRP at a transcriptomic level was evaluated by RT-PCR. The protein level was then evaluated, as reported in paragraph 4.6 and **Fig.4.9** The IL-1β gene expression was taken as a reference, bearing in mind that a previous study reported that the TNF-α mRNA life span is short, even in the presence of a high TNF-α cytokine release (Mijatovic et al., 2000).

After 24 hours of macrophage LPS (10 ng/mL) or mCRP (100 μg/mL) stimulation, the IL-1β gene expression level was assessed as shown in **Fig.4.8**. The result here reported confirm that a concentration of 100 μg/mL the mCRP increased the IL-1β gene expression at 24 hours.

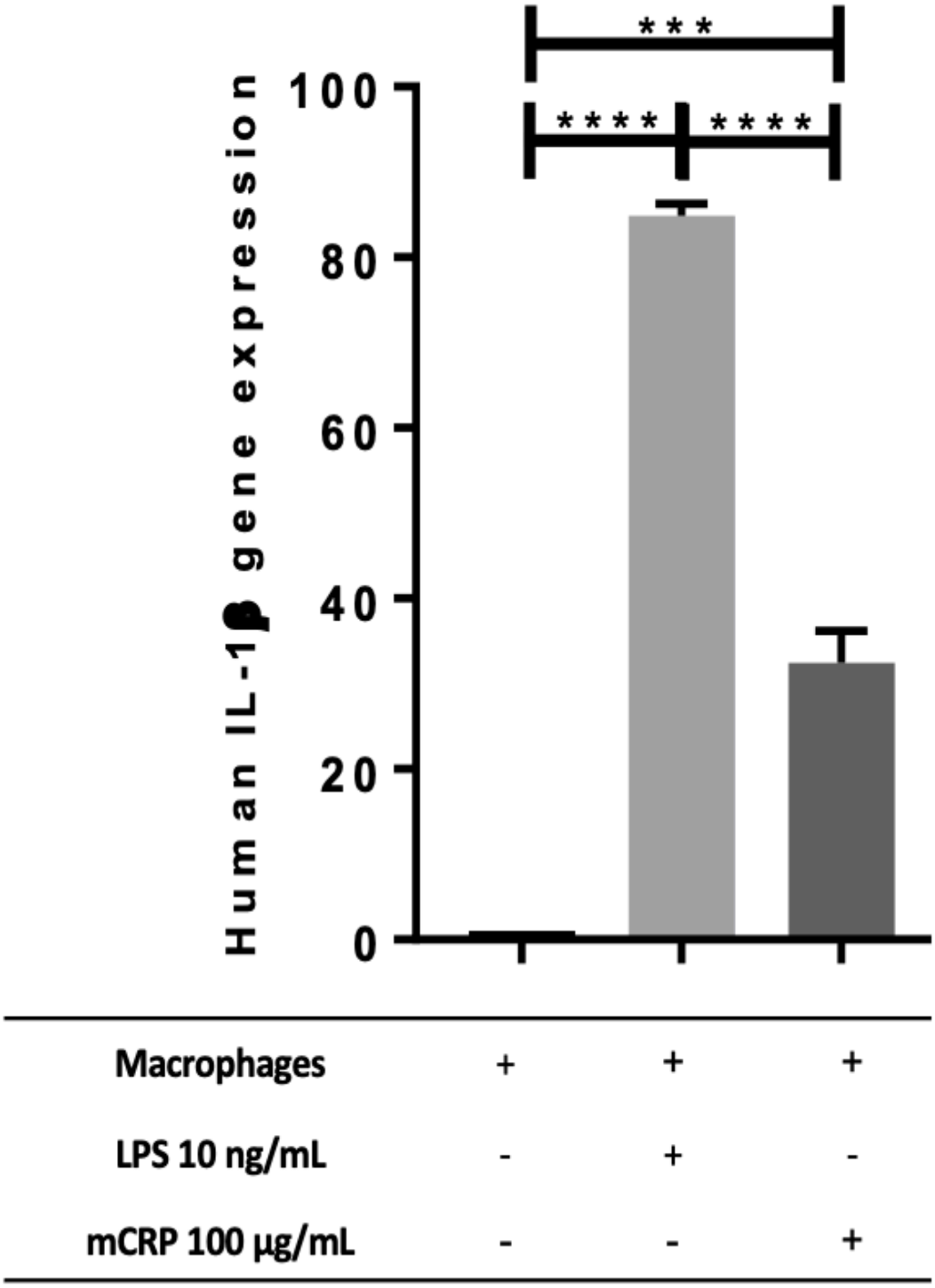


Fig. 4.8. Monomeric-CRP increases the IL-1β mRNA expression. After differentiation, the monocyte-derived macrophages were stimulated by LPS (10 ng/mL) or mCRP (100 µg/mL) for 24 hours. The IL-1β gene expression was quantified by both the TaqMan™ Fast Advanced Master Mix and TaqMan® Gene Expression Assays. The results are shown as the mean ± SD. ****p-value< 0.0001, ***p-value < 0.005. The One-way ANOVA test, followed by the Tukey Post-doc test with a 99.9% CI and a α error 0.01%, evidenced a statistically significant difference (p-value<0.002) between the unstimulated macrophage and the mCRP group when both groups were tested at 24 hours. The ANOVA test also indicated a higher statistical difference (p-value<0.0001) between the positive control and the mCRP group after 24 hours. (n=3).

4.6 mCRP promotes a pro-inflammatory in U937-derived macrophages.

After observing that mCRP exposure increases IL-1β at a transcriptomic level, the protein expression was also assessed. Four different cytokines (TNF-α; IL-6; IL-10; and IFN-γ) were examined and compared with the unstimulated macrophages. TNF-α was tested as it is has been reported that this pro-inflammatory cytokine plays a pivotal role in several inflammatory diseases and is principally released by macrophages (Parameswaran and Patial, 2010). Whilst IL-6 was selected for its relevant role in several situations relating chronic inflammation and conditions with endothelial cell dysfunction (Barnes et al., 2011). IL-10, which has anti-inflammatory effects, was evaluated as it as it is released by M2 macrophages that bind to the IL-10R present in M1 macrophages (Shi et al., 2019) . This is able to reduce the release of both TNF-α and IL-6 pro-inflammatory cytokines (Moore et al., 2001). In addition to the other cytokines, IFN-γ was also assessed to evaluate its inflammatory effect in the model studied, even if it has been reported that IFN-γ is not released by macrophages (Schoenborn and Wilson, 2007; Kulkarni et al., 2016).

The results showed that macrophages had a very strong pro-inflammatory activity when stimulated with 100 µg/mL of mCRP for 24 hours **Fig. 4.9.**

As shown in **Fig. 4.9A.** TNF-α protein concentrations among the unstimulated macrophage group, the positive control group (LPS 10 ng/mL) and the group stimulated with mCRP (100 µg/mL) were evaluated after 24 hours. There was a statistically significant difference between: the unstimulated macrophage group and the positive control; the unstimulated macrophage group and the mCRP group; the positive control and the mCRP group.

Fig. 4.9B shows the IL-6 protein concentrations in the unstimulated macrophage group, the positive control group (LPS 10 ng/mL) and the group stimulated with mCRP (100 µg/mL), evaluated after 24 hours. There was a statistically significant difference between: the unstimulated macrophage

group and the positive control group; the unstimulated macrophage group and the mCRP group; the positive control and the mCRP group.

mCRP stimulation did not increase the IL-10 concentration. No statistical differences (p-value = 0.1519) were observed between the unstimulated macrophage group and the mCRP group (1523 *vs* 132.6 pg/mL respectively) (**Fig. 4.10A**.). **Fig. 4.10A** shows the statistically significant difference (p-value<0.0001) between the positive control group and the mCRP group (3872 *vs* 132.6 pg/mL, respectively).

The role macrophages play in IFN-γ cytokine production was also evaluated. In agreement with literature (Schoenborn and Wilson, 2007; Kulkarni et al., 2016), the macrophages were unable to release any IFN-γ cytokines (**Fig. 4.10B**)**.** This is most likely due to the fact that macrophages can only release IFN-γ in response to both IL-12 and IL-18 stimulation, as suggested by Darwich et al. (2009).

The results shown in **Fig. 4.9** and **Fig. 4.10** showed that stimulation with mCRP, at a concentration of 100 µg/mL, was able to increase the TNF-α production and the IL-6 protein concentration. However, the results also indicated that mCRP may also partially reduce the IL-10 concentration, without increasing the IFN-γ protein concentration (**Fig.4.10B**).

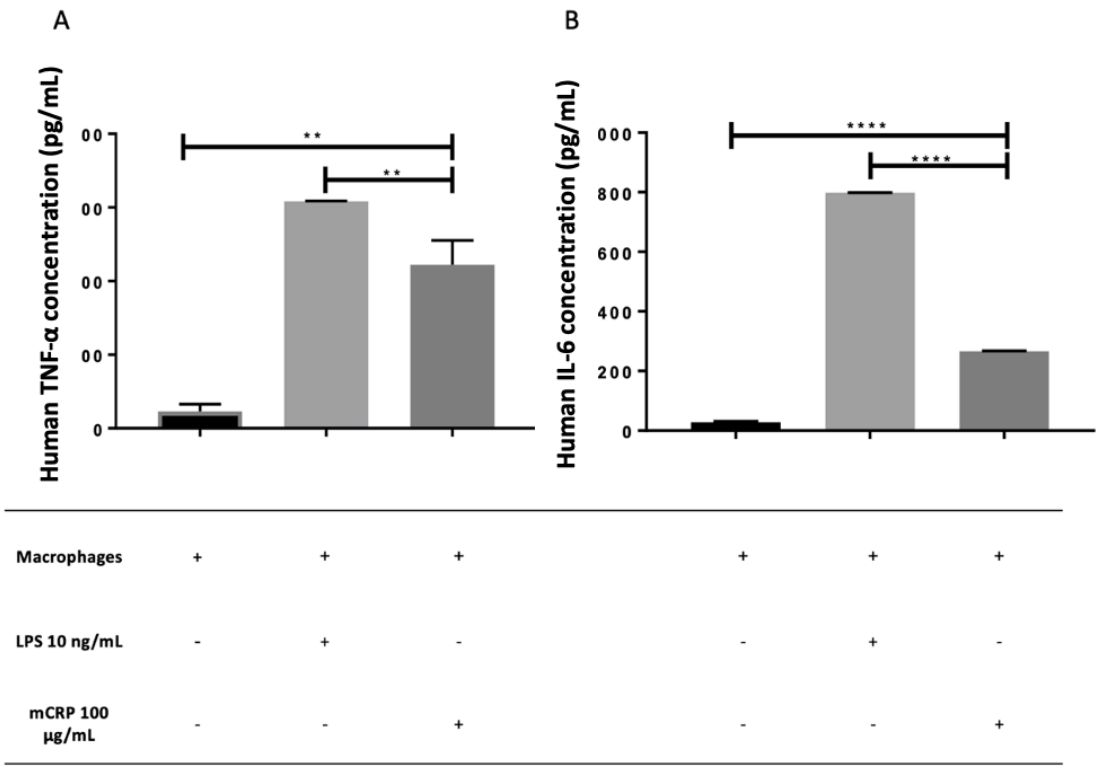


Fig. 4.9. mCRP increases both TNF-α and IL-6. After differentiation, the macrophages were treated with positive control (LPS 10 ng/mL) or mCRP (100 µg/mL) for 24 hours. The TNF-α (A) and IL-6 (B) production was quantified by ELISA kits (R&D System). The results are shown as the mean ± SD. ****p-value <0.0001, **p-value ≤0.05. Monomeric-CRP increased the concentration of TNF-α (A) and IL-6 (B) pro-inflammatory cytokines. Fig (A) The One-way ANOVA test, with a 95% CI and a 5%α error, confirmed a statistically significant difference (p-value<0.0044) between the unstimulated macrophage group and the mCRP group (113.8 vs 1110 pg/mL respectively). The ANOVA also demonstrated a statistical difference (p-value<0.042) between the positive control group and the mCRP group (1540 vs 1110 pg/mL respectively). Fig (B) The One-way ANOVA test, with a 95.% CI and a 5%α error confirmed the statistical difference (p-value<0.0001) between the unstimulated macrophage group and mCRP group (27.86 vs 266 pg/mL, respectively) as well as a statistically significant difference (p-value<0.0001) between the positive control group and the mCRP group (798.4 vs 266.1 pg/mL, respectively) (n=3).

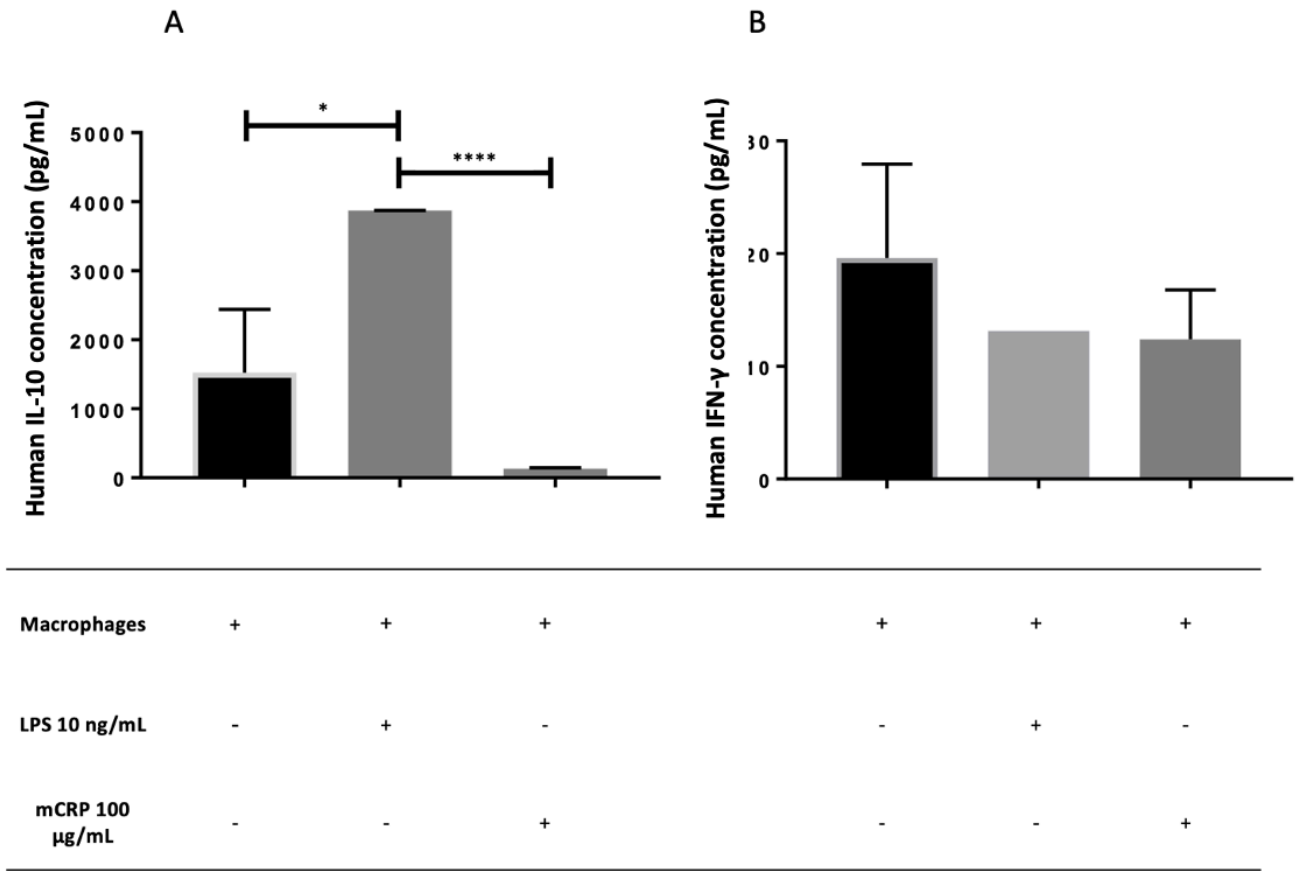


Fig. 4.10. IL-10 and IFN-γ levels are not susceptible to mCRP. After 72 hours of PMA (50 ng/mL) treatment, the macrophages were stimulated with positive control (LPS 10 ng/mL) or mCRP (100 µg/mL) for 24 hours. The IL-10 (A)-and IFN-γ (B) production was quantified by ELISA kits (R&D System). The results are shown as the mean ± SD. ****p-value <0.0001, *p-value <0.10. Fig (A) The One-way ANOVA test, with a 95 % CI and a 5%α error confirmed a statistically significant difference (p-value <0.0001) between the positive control group and the mCRP group (3872 vs 132.6 pg/mL, respectively). Fig (B) shows that the macrophages were unable to release any IFN-γ cytokines (<20 pg/mL). (n=3).

4.7 FCγR antibodies did not induce pro-inflammatory effects on U937 macrophages.

After confirming that mCRP had both biological and pro-inflammatory effects, the next step was that of clarifying whether blocking the FCγRs would have led to a reduced mCRP biological and pro-inflammatory activity. As aforementioned in the introduction paragraph, mCRP pro-inflammatory proprieties were abolished when FCγI (CD64), FCγIIa (CD32) and FCγIII (CD16) were blocked (T. Khreiss et al., 2004; Eisenhardt et al., 2009b; Thiele et al., 2014a). This prompted the assessment of the correlation between mCRP pro-inflammatory activity and the FCγRs. However, to address the hypothesis that mCRP pro-inflammatory proprieties were FCγR mediated, it was necessary to evaluate whether the FCγR antibodies had pro-inflammatory effects on U937 macrophages (ELISA test).

As detailed in paragraph 4.6, TNF-α, IL-6 and IL-10 were tested in this experiment. The antibody concentration (100 μg/mL) was the same as that used for mCRP (previously reported in paragraph 4.6). The macrophages were stimulated by the anti-FCγRs antibodies for 24 hours, which was the best pre-incubation time-point for all (SMIs) to inhibit mCRP pro-inflammatory activity (see chapter 5, paragraph 5.4).

As shown in **Fig. 4.11A**, the TNF-α protein concentrations in the unstimulated macrophage group, the positive control group and the groups stimulated with anti-FCγRs antibodies (anti-CD16, anti-CD32, and anti-CD64) 24 hours after stimulation were evaluated. No statistically significant difference was observed among the unstimulated macrophage group and the FCγR antibody groups, with the exception of the unstimulated macrophage group and the anti-FCγRs (anti-CDs groups), were compared with the positive control group.

As shown in **Fig.4.11B,** the IL-6 protein concentrations in the unstimulated macrophage group, the positive control group and the groups stimulated with

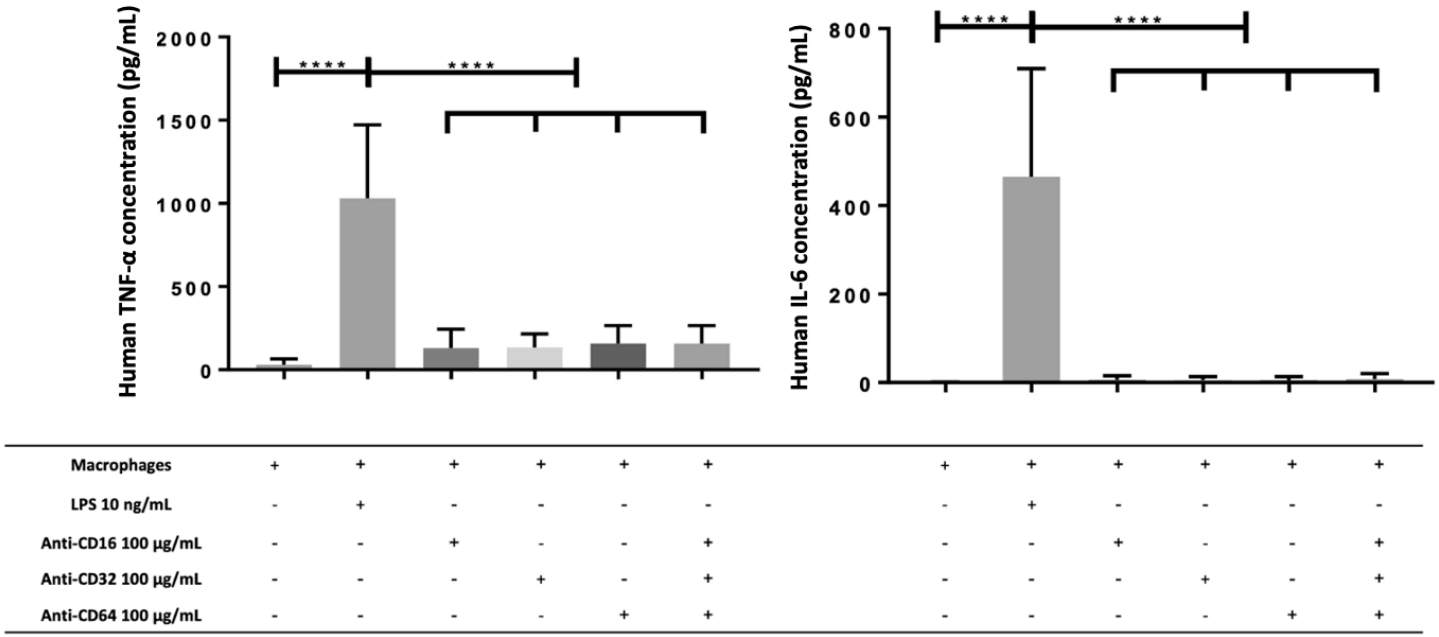
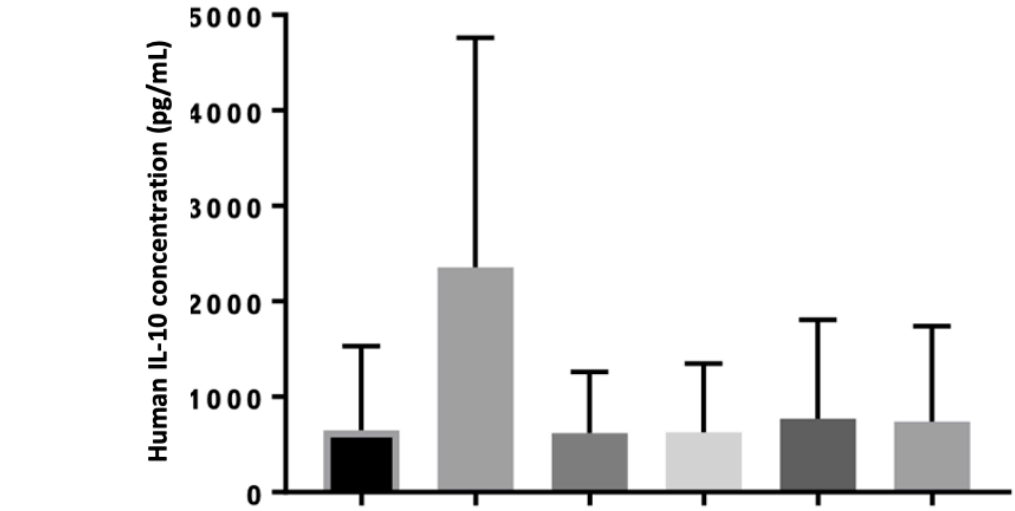


Fig. 4.11. **FCγR antibodies did not induce pro-inflammatory effects on U937 macrophages.** After differentiation (PMA 50 ng/mL for 72 hours), the macrophages were stimulated by LPS at 10 ng/mL (positive control) or anti-FCγRs antibodies (anti-CD16, anti-CD32, and anti-CD64) at a concentration of 100 µg/mL, for 24 hours. The TNF-α (A)-and IL-6 (B) production was quantified by ELISA kits (R&D System). Results are showed as the mean ± SD. ****p-value <0.0001. The positive control increased the TNF-α (A) and IL-6 (B) pro-inflammatory cytokine concentrations. Fig (A) The One-way ANOVA test, followed by the Tukey Post-doc test with a 99.9% CI and α error fixed at 0.001% revealed no statistical differences (p-value= 0.8779) between the unstimulated macrophage group (31.01 ± 14.33 pg/mL) and the group treated with all anti-FCγRs antibodies pooled together (157.8 ± 44.3 pg/mL) which presented with a higher value. The only statistically significant finding (p-value <0.0001) was observed when the unstimulated macrophage group and the positive control group (31.01 vs 1030 pg/mL respectively), along with the positive control group and the FCγRs groups, were compared (p-value<0.0001). Fig (B) The One-way ANOVA test, followed by the Tukey Post-doc test, with a 99.9% CI and α error fixed at 0.01% showed no statistically significant differences (p-value 0.9999) between the unstimulated macrophage group (0.2088 ± 0.2088 pg/mL) and the anti-FCγRs antibodies pooled together (8.009 ± 5.119 pg/mL) which presented with a higher value. The only statistically significant difference (p-value <0.0001) was observed when the unstimulated macrophage group and the positive control group (0.2088 vs 465.1 pg/mL respectively) were compared, or when the positive control group was compared to the anti-FCγRs antibody groups (p-value <0.0001). (n=3)

anti-FCγRs antibodies (anti-CD16, anti-CD32, and anti-CD64) were compared 24 hours after stimulation. The results showed no statistically significant difference among the unstimulated macrophage group and the FCγR antibody groups. The only statistically significant difference emerged when the unstimulated macrophage group and the anti-FCγRs groups (anti-CDs groups), were compared with the positive control group (LPS 10 ng/mL).

As shown in **Fig. 4.12,** IL-10 protein concentrations in the unstimulated macrophage group, the positive control group and the group stimulated with anti-FCγRs antibodies (anti-CD16, anti-CD32, and anti-CD64) after 24 hours of stimulation, were evaluated. No statistically significant difference was observed among the unstimulated macrophage group and the anti-FCγR antibody groups. Furthermore, no statistical differences were reported for IL-10 cytokines when the anti-FCγR antibody groups were compared to the positive control group.



Macrophages	+	+	+	+	+	+
LPS 10 ng/mL	-	+	-	-	-	-
Anti-CD16 100 µg/mL	-	-	+	-	-	+
Anti-CD32 100 µg/mL	-	-	-	+	-	+
Anti-CD64 100 µg/mL	-	-	-	-	+	+

Fig. 4.12. **FCγR antibodies did not increase the release of IL-10 anti-inflammatory cytokine.** After differentiation (PMA 50 ng/mL for 72 hours), the macrophages were stimulated by LPS at 10 ng/mL (positive control) or anti-FCγRs antibodies (anti-CD16, anti-CD32, and anti-CD64) at a concentration of 100 µg/mL, for 24 hours. The production of IL-10 was quantified by ELISA kits (R&D System). LPS stimulation at 10 ng/mL increased the IL-10 anti-inflammatory cytokine concentration. The One-way ANOVA test, followed by the Tukey Post-doc test, with a 99.9% CI and an α error fixed at 0.01% showed no statistically significant differences ($p > 0.9999$) between the unstimulated macrophage group (647 ± 361.8, pg/mL) and the anti-FCγRI (anti-CD64) group (766.7 ± 425 pg/mL), the group with the highest value. The One-way ANOVA test also confirmed that there were no statistical differences (p-value= 0.1940) when the control groups were compared to the anti-CDs groups. (n=3).

4.8 The FCγRs antibodies did not activate any signalling pathways potentially associated to a pro-inflammatory response.

After having confirmed that the anti-CD antibodies under study were not able to increase the cytokines release, it became essential to prove that the anti-CD antibodies did not activate any signalling pathways that may be have been associated to a pro-inflammatory response. As detailed in chapter 1, paragraph 1.1.7.10 MAPK signalling pathways are triggered during inflammation, cancer and IS (Kaminska, 2005; Wang et al., 2006; Del Reino et al., 2014; Maik-Rachline et al., 2018). NF-KB is a transcription factor (Hayden and Ghosh, 2008) activated by viruses, bacterial toxins (including LPS), UV light, oxidative stresses, inflammatory stimuli and cytokines (Shih et al., 2015). It has been reported that when macrophages were exposed to LPS these cell up-regulated the MAPKp38/JNK/NF-KB intrinsic pathways and increased the production of pro-inflammatory cytokines, such as IL-1β and TNF-α (de Oliveira et al., 2017). The research data have shown that mCRP is able to raise the IL-6 concentration when both MAPKp38 and NF-KB signalling pathways are involved (Li et al., 2014). Confirming that activation of both MAPKp38 and NF-KB transcription factor increases the production of some pro-inflammatory cytokines (Yenari and Han, 2006; Jin et al., 2013; Li et al., 2014). Bearing in mind that both these pathways are involved in inflammation, the next step to follow was that of evaluating whether the FCγR antibodies had the potential to activate a pro-inflammatory response.

The following experiment used various treatment stimulation methods to evaluate the anti-FCγR pro-inflammatory antibody effect (anti-CD16, anti-CD32 and anti-CD64). Cell lysate, obtained from unstimulated macrophages, was used as a negative control. The LPS stimulated U937 macrophages (10 ng/mL), were used as a positive control (column 2). The activation effect on inflammatory pathways was evaluated by FCγRIII (anti-CD16), FCγRII (anti-CD 32) and FCγRI (anti-CD64) antibody stimulation. Inflammatory pathway activation was evaluated without LPS (**Fig 4.13**)**.** All the antibodies were used singularly and combined (**Fig. 4.13**). As reported in **Fig. 4.13,** none of the selected anti-FCγR antibodies were able to activate any signalling pathways that may have been associated to an inflammatory response. The results

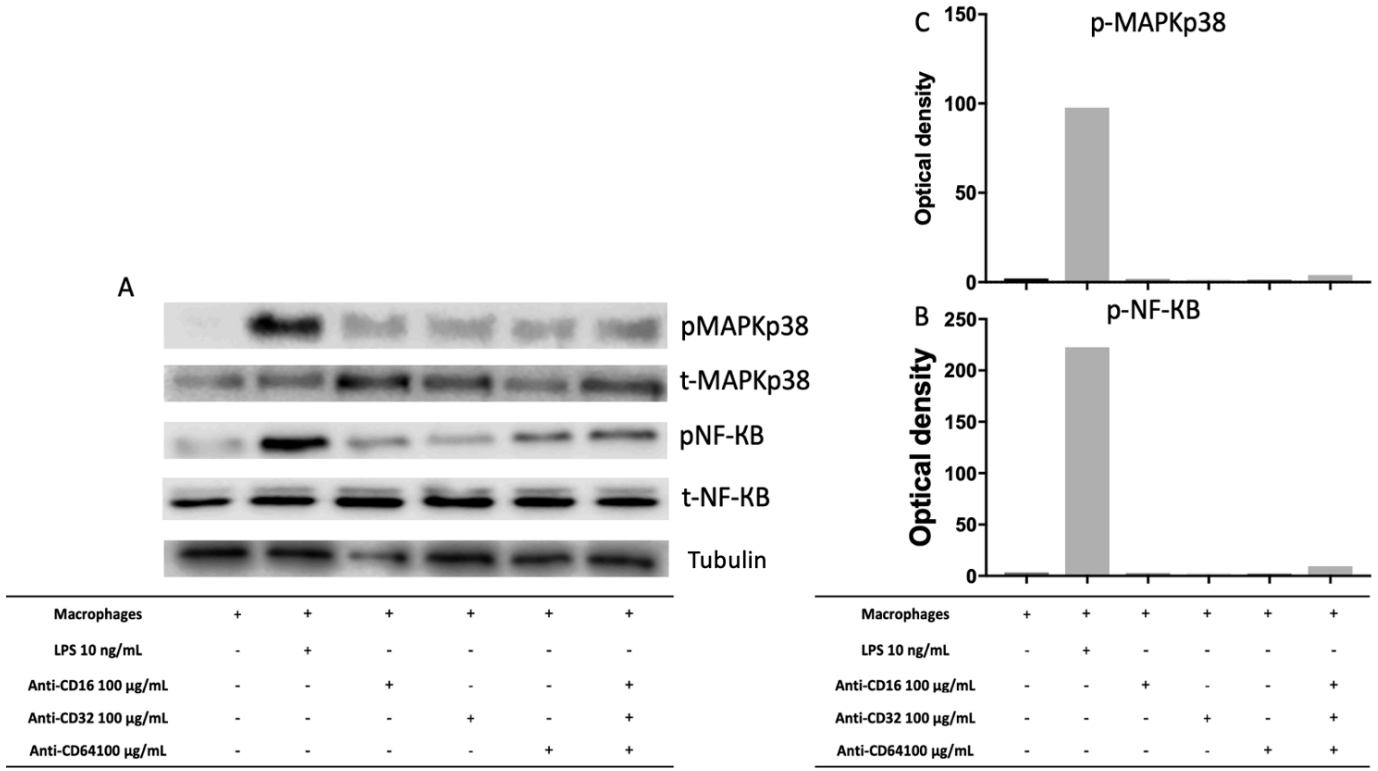


Fig 4.13. The FCγRs antibodies did not activate MAPKp38 and NF-KB signalling pathways potentially associated to a pro-inflammatory response. Anti-FCγR (anti-CD16, anti-CD32, and anti-CD64) antibody stimulation did not lead to an increase in the selected phosphoproteins MAPKp38 and NF-KBp65 which are known to be related to inflammation. After 3 days (72 hours) of PMA stimulation (50 ng/mL), the monocyte-derived macrophages were stimulated by LPS (10 ng/mL) anti-FCγRIII (CD16), anti-FCγRII (CD32) and the anti-FCγRI (CD64) antibody all at concentrations of 100 µg/mL. After discarding the medium, the macrophages were washed twice with PBS for 2 minutes followed by the addition of 250 µl of lysis buffer, containing a protease inhibitor cocktail, as well as a phosphatase inhibitor cocktail. Lane 1, unstimulated macrophage; Lane 2, LPS (10 ng/mL); Lane 3, anti-CD16 antibody (100 µg/mL), Lane, 4 anti-CD32 antibody (100 µg/mL), Lane5, anti-CD64 antibody (100 µg/mL), Lane 6, anti-CD163264 antibodies (100 µg/mL). Fig (A) shows phospho-MAPKp38 expression and phospho-NF-KB expression. Fig (B) Graph shows the phospho-NF-KB expression. Fig (C) Graph shows the phospho- MAPKp38 expression (n=1).

showed that no antibodies were likely to increase either MAPKp38 phosphorylation or NF-KBp65 phosphorylation.

The ELISA assay also showed that when macrophages were incubated with antibodies, in combination with the LPS (10 ng/mL for 24 hours), there were no statistically significant differences (p-value=0.6858) in terms of TNF-α release.

The TNF-α pro-inflammatory cytokines was selected based on the same hint expressed in chapter 4 paragraph 4.6. None of the anti-FCγR antibodies showed any anti-inflammatory properties. These results are summarised in **Fig. 4.14.**

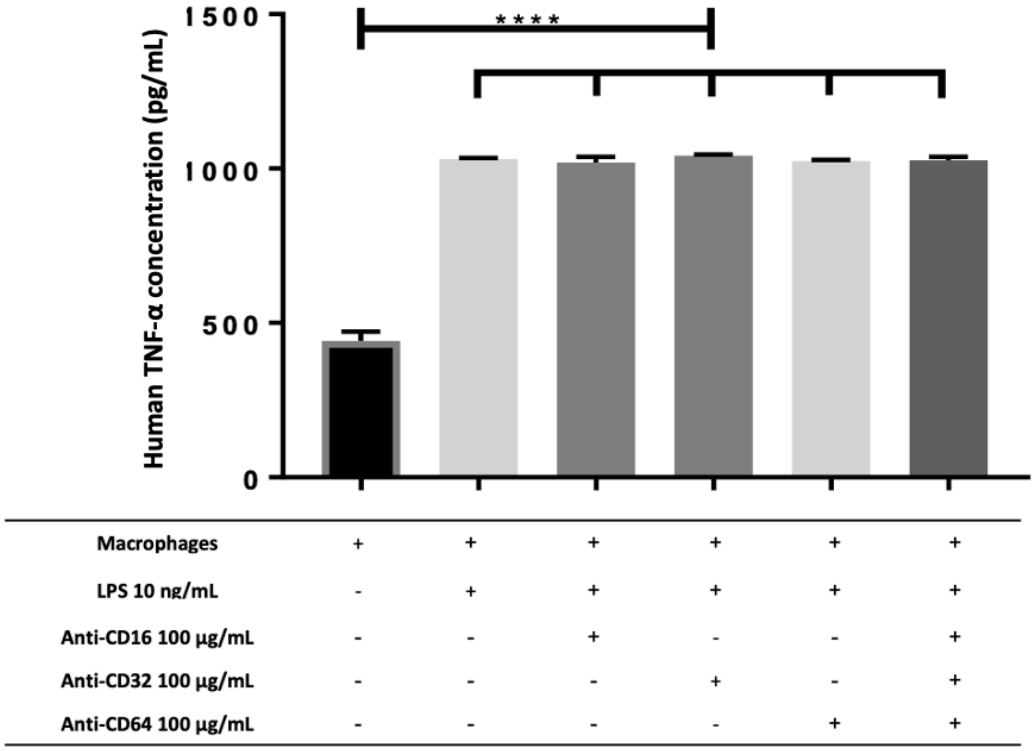


Fig. 4.14. Anti-FCγR antibodies are unable to inhibit LPS pro-inflammatory activity. After 72 hours of PMA stimulation (50 ng/mL), the monocyte-derived macrophages were pre-treated with anti-FCγRIII (CD16), anti-FCγRII (CD32) and the anti-FCγRI (CD64) antibody at a concentration of 100 µg/mL for 2 hours and then stimulated with LPS (10 ng/mL) for 24 hours. Only LPS (10 ng/mL) for 24 hours (i.e., without antibodies) was used as a positive control. The pro-inflammatory cytokine TNF-α was estimated by ELISA kits (R&D System). The results are shown as the mean ± SD. ****P-value <0.0001. There was a substantial increase in TNF-α in the macrophages after LPS stimulation at 10 ng/mL for 24 hours. The One-way ANOVA test with a 95 % CI and a 5%α error, confirmed the statistically significant difference (p-value <0.0001) among the unstimulated macrophage group and all the groups stimulated by LPS. There was no statistically significant difference when only the LPS stimulated groups were compared (p-value = 0.6858). (n=3)

4.9 The FCγRs antibodies did not block mCRP's pro-inflammatory activity.

After confirming that the FCγR antibodies did not contribute to triggering any pro or anti-inflammatory activity, a similar experiment was carried out by replacing LPS with the mCRP. TNF-α, and IL-6 protein levels were evaluated by the ELISA test, based on the same information in paragraph 4.6. Conversely, IL-10 and IFN-γ were not evaluated as there was no statistical difference in experiment 4.6. Therefore, this experiment aimed at assessing whether the mCRP pro-inflammatory activity could be mediated by FCγRs. The same antibody concentrations and time-points as in the experiment in paragraph 4.7. were adopted. Aimed at reducing the mCRP pro-inflammatory activity, it was assessed whether the FCγRs antibodies were capable of interfering with the binding site between mCRP and FCγRs.

As shown in **Fig. 4.15A,** the TNF-α protein concentrations in the unstimulated macrophage group, the positive control group and the group stimulated with mCRP (pre-treated or not) with anti-FCγRs (anti-CD16, anti-CD32 and anti-CD64) antibodies, were evaluated after 24 hours. No statistically significant difference was observed among the positive control group and the mCRP stimulated groups. Nor was there any statistical difference amongst the mCRP and anti-FCγR (anti-CD16, anti-CD32 and anti-CD64) antibody pre-stimulated groups.

As shown in **Fig.4.15B,** the IL-6 protein concentrations in the unstimulated macrophage group, the positive control group and the group stimulated with mCRP (pre-treated or not) with anti-FCγRs (anti-CD16, anti-CD32, and anti-CD64) antibodies for 24 hours, were evaluated and compared. There was no statistical significance when the positive control group was compared to the mCRP stimulated groups or when the mCRP group was compared to the anti-FCγR (anti-CD16, anti-CD32, and anti-CD64) antibody pre-stimulated groups.

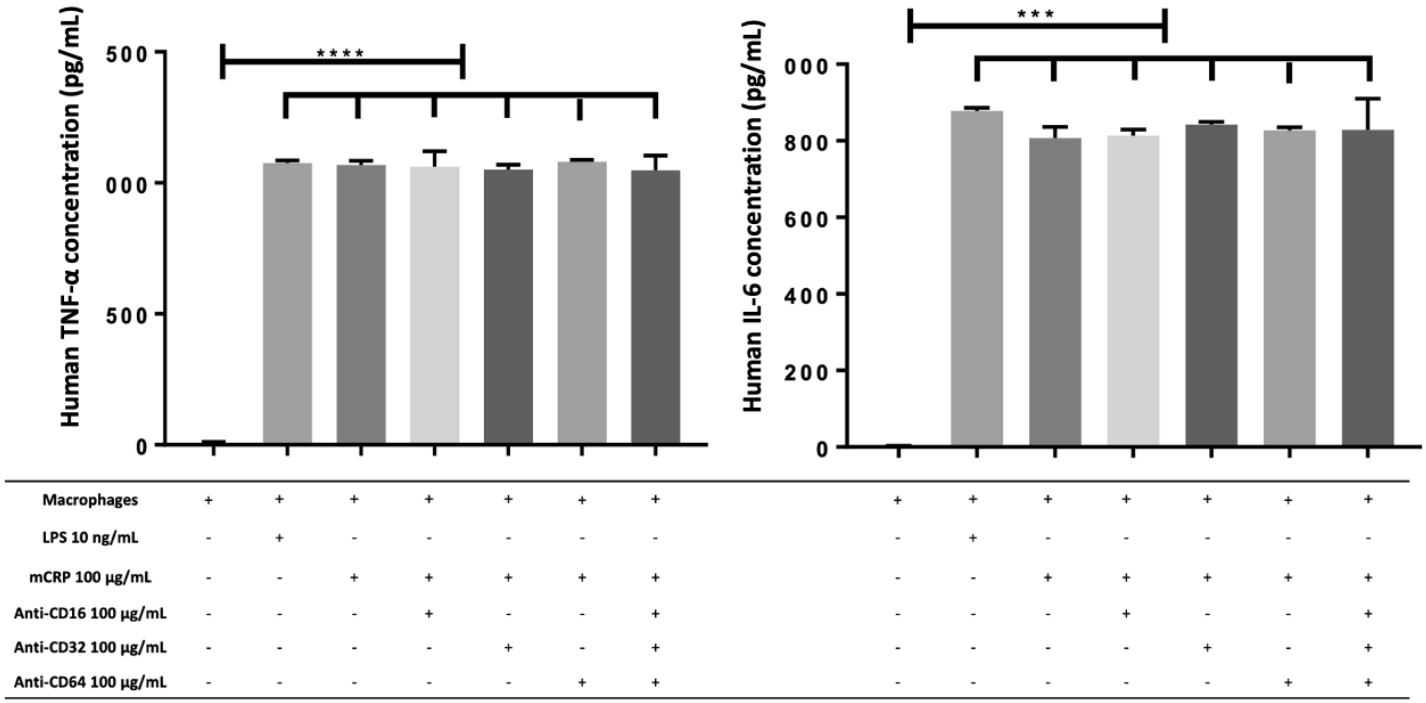


Fig 4.15. The FCγRs antibodies did not block mCRP's pro-inflammatory activity. PMA exposure (50 ng/mL) monocyte-derived macrophages were pre-treated with anti-FCγRIII (CD16), anti-FCγRII (CD32) and the anti-FCγRI (CD64) antibody at a concentration of 100 µg/mL for 2 hours followed by 24 hours of mCRP (100 µg/mL) stimulation. LPS (10 ng/mL for 24 hours) stimulation was used as a positive control. The pro-inflammatory TNF-α and IL-6 cytokine estimation was made by ELISA kits (R&D System). The results are shown as the mean ± SD. ****p-value<0.0001 *p≤ 0.05. Fig (A) a statistically significant increased TNF-α production was observed in macrophages after mCRP stimulation at 100 µg/mL. The One-way ANOVA test, with Tukey's post-hoc test with a 99.9% CI and an α error fixed at 0.01%, confirmed the significant difference (p-value <0.0001) between the TNF-α expression level in the unstimulated macrophage group compared to the group with macrophages stimulated by mCRP (6.167 vs 1,068 pg/mL, respectively). However, none of the anti-FCγR antibodies under study were able to block mCRP pro-inflammatory activity (p-value =0.2708). Fig (B) The One-way ANOVA test, with Tukey's post-hoc test with a 99.9% CI and α error fixed at 0.01%, confirmed the significant difference (p-value <0.0001) in the IL-6 expression level between the unstimulated macrophage group and the macrophage group stimulated by mCRP (1.531 vs 806.8 pg/mL, respectively). However, none of the anti-FCγR antibodies under study were able to block the mCRP pro-inflammatory activity (p-value=0.8816). (n=3)

4.10 Discussion.

As aforementioned, in the event of an MI or IS, the priority is that of restoring the blood flow (Pisters and Lip, 2013; Howard, 2016). Several authors have reported an accumulation of nCRP, mCRP and macrophages during these pathophysiological events (Slevin et al., 2010; Chiba and Umegaki, 2013; Swirski and Nahrendorf, 2013; McFadyen et al., 2018), which might trigger macrophage pro-inflammatory activity. Therefore, it was assessed whether the macrophages were able to release pro-inflammatory cytokines when exposed to mCRP. Furthermore, it was determined whether the mCRP pro-inflammatory activity was linked to mCRP bound to the FCγRs.

The data obtained in this chapter suggest that monocytes are capable of a rapid duplication rate (**Fig.4.1**). However, these cells were unable to release the pro-inflammatory TNF-α cytokines after stimulation by LPS at 100 ng/mL (**Fig. 4.6**)**.** Following a similar experimental design, LPS was replaced by mCRP. Previous studies have reported that mCRP could bind to αvβ3 and α4β1 integrins, as well as to FCγRI and FCγRIII receptors on monocytes (Fujita et al., 2014; Thiele et al., 2014a). Although some authors reported an increase (more than 1.5-fold) in NO and iNOS production (Sproston et al., 2018), the data in this research showed that monocytes were not capable of increasing the TNF-α concentration. This datum is in agreement with other literature reports where it is stated that the increase in NO levels are associated to an increase in TNF-α and IL-6 only if the monocytes are differentiated into macrophages (Wang et al., 1997; Hur et al., 2004).

As previously described in chapter 1 paragraph 1.2.2.1 and in the introduction chapter, monocytes are immune system cells able to differentiate into an initial resting macrophage (M0) phenotype (Zhao et al., 2017). *In vitro* differentiation can be obtained by PMA stimulation, which has been shown to differentiate the U937 monocytes into macrophages (Sproston et al., 2018) without influencing their polarization (Passmore et al., 2001; Song et al., 2015). In agreement with literature, the data herein reported confirm that PMA stimulation is able to lead to a significant increase in the Human-CD11c

macrophage markers (Sproston and Ashworth, 2018; Li et al., 2017). Furthermore, after macrophage differentiation, it was observed that these cells were also able to release both pro and anti-inflammatory cytokines (TNF-α, IL-6, IL-10) after LPS stimulation (**Fig. 4.6**)**,** in line with other authors (Wang et al., 1997; Hida et al., 2000; H. J. Hur et al., 2004; Tucureanu et al., 2018).

After differentiating into a resting M0 macrophage phenotype state, macrophages are able to polarize into a pro (M1) or anti (M2) inflammatory phenotype (Orekhov et al., 2019). This polarization might be dependent on the presence of Pathogen Associated Molecular Patterns (PAMP) and/or Damage-associated molecular patterns (DAMPs) released upon cellular stress or tissue injury (Liu et al., 2017a). The past few years have witnessed publications reporting that nCRP could polarise macrophages from M2 to M1 when they bind to FCγRII and FCγRI receptors (Devaraj and Jialal, 2011). However, more recent investigation has indicated that this maturation and polarisation are led by mCRP (Trial et al., 2016). If the maturation and polarisation towards a pro-inflammatory phenotype were led by mCRP, it might then also be suggested that mCRP may be the player responsible for the increase and release of the pro-inflammatory cytokines TNF-α and IL-6, without increasing the release of the anti-inflammatory cytokine IL-10 (which is synthesized specifically by the macrophage M2 phenotype) (Moore et al., 2001).

It was observed that mCRP had a direct biological activity on U937 monocyte-derived macrophages. Indeed, the macrophages were able to up-regulate the IL-1β (**Fig 4.8**) gene expression level when stimulated by mCRP at a concentration of 100 µg/mL for 12 and 24 hours. This up-regulation was particularly high when the macrophages were stimulated by mCRP for 12 hours (**Fig 4.8**)**.** Furthermore, it was observed that these biological effects are directly related to an increase of both TNF-α and IL-6 (**Fig. 4.9**). However, differently to LPS, mCRP was unable to increase the IL-10 anti-inflammatory cytokine release (**Fig. 4.10**).

The possibility that the U937-derived macrophages were able to increase TNF-α and IL-6 without increasing the release of IL-10 anti-inflammatory cytokines not only confirms the potential involvement of this protein in macrophages polarisation towards a pro-inflammatory phenotype (Trial et al., 2016), but also suggests that mCRP is capable of boosting inflammation, thus substantially altering the micro-environment (Singh et al., 2006), leading to serious detrimental clinical complications (Slevin et al., 2015; Krayem et al., 2017). An IS event is an example. The presence mCRP after an IS accident (Slevin et al., 2010) has been associated with the probability of developing future neurological clinical complications, such as dementia and Alzheimer (Desmond et al., 2002; Slevin et al., 2015; Slevin et al., 2017). Literature confirms that the macrophage M2 phenotype plays an important role after an IS event (Hu et al., 2012). Indeed, after an IS event the macrophage M2 phenotype can originate through several mechanisms. For example, Gliem et al. (2012) confirmed that, following a transient middle cerebral artery occlusion (tMCAO), the recluted pro-inflammatory classical monocytes could be polarised into the non-classical monocyte phenotype M2. Other studies demonstrated that, when monocytes are recruited through the CC-chemokine receptor 2 (CCR2), they differentiated into macrophages leading to the polarisation of macrophages from the pro-inflammatory (M1) to the anti-inflammatory phenotype (M2) (Gliem et al., 2016). This implies an increase in the transforming growth factor (TGF-β1), VCAM1, IL-10, brain-derived neurotrophic factors (BDNF), insulin-like growth factor (IGF-1) and vascular endothelial growth factor (VEGF) levels, all of which are associated with a better long-term spontaneous recovery (David and Kroner, 2011; Dobolyi et al., 2012; Wattananit et al., 2016; Chernykh et al., 2016). However, should this recruitment take place along with the concomitant mCRP-brain accumulation (Slevin et al., 2010), it maybe that the mCRP interferes with the maturation and/or polarisation from M0 or M1 to the M2 phenotype. This would induce a substantial reduction in anti-inflammatory cytokines and the release of the trophic factor, risking the facilitation of the appearance of serious clinic neurological consequences, as reported in two recent studies (Slevin et al., 2015; Slevin et al., 2017). These hypotheses may well be of clinical value

considering that macrophage polarisation has recently been hypothesised as a *novel therapy* against stroke (Kanazawa et al., 2017).

The possibility that mCRP facilitates and increases the release of both the TNF-α and IL-6 protein concentration, allows us to postulate which type of inflammatory pathway the mCRP is able to trigger. As aforementioned in paragraph 4.8, MAPKs and NF-KB are signalling pathways that activate during acute and chronic inflammation conditions (Wang et al., 2006; Hayden et al., 2006a; Del Reino et al., 2014; Shih et al., 2015; Maik-Rachline et al., 2018). Once these pathways are activated, macrophages are able to increases the pro-inflammatory cytokine production, such as IL-1β and TNF-α (Jin et al., 2013; Yang et al., 2014b). For example, some studies have reported that in LPS-activated RAW 264.7 macrophages and mice microglia cells, up-regulation of MAPKp38/JNK/NF-KB pathways increase the production of pro-inflammatory mediators, such as IL-1β and TNF-α (de Oliveira et al., 2017; Lim et al., 2018). Literature also confirms that there is an elevated increase in pro-inflammatory cytokines when NF-KB nuclear translocation takes place in the M1 pro-inflammatory phenotype (Wang et al., 2014). When reflecting on the data and information obtained in this study, along with that of literature, it comes to mind that another interesting issue could be that of evaluating whether mCRP is capable of activating the ERK, JNK, MAPKp38 and NF-KB pathways. As mCRP is able to polarize macrophages towards to the pro-inflammatory M1 phenotype (Trial et al., 2016), with the direct consequence of an increase in pro-inflammatory cytokines (result paragraph 4.6), it might also be possible that mCRP is able to increase the release of the pro-inflammatory mediator through the activation of MAPKp38/JNK/NF-KB pathway.

This hypothesis could also be supported by the fact that mCRP is known to increase both NO and iNOS levels in monocytes (Sproston et al., 2018). This is associated with the activation of MAPKp38 as a fundamental step to produce

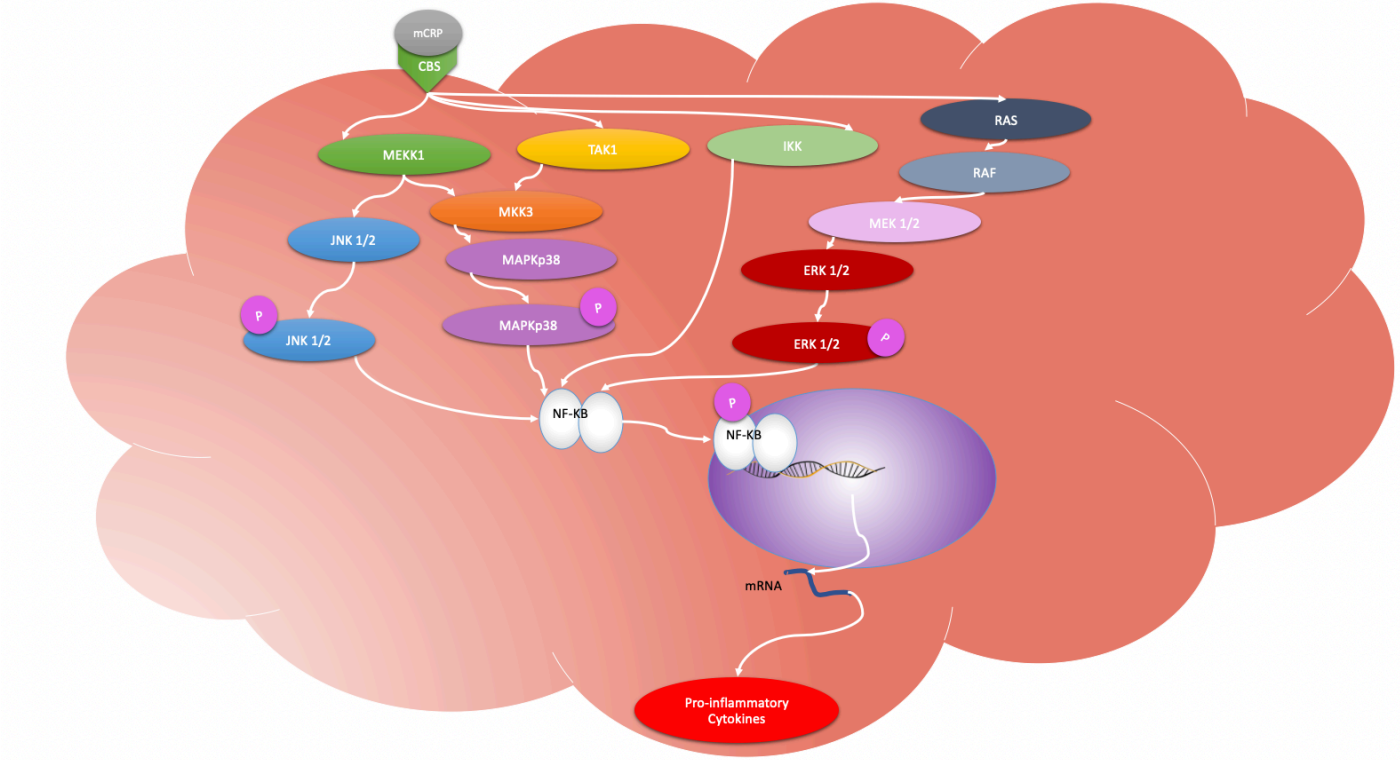


Fig 4.16. mCRP might increase the release of the pro-inflammatory mediator through the activation of MAPKp38/JNK/NF-KB pathway. Both MAPK and NF-KB are signalling pathways activated during acute and chronic inflammation. Studies have already reported that when both MAPKs and NF-KB are activated these signalling pathways are able to increase the pro-inflammatory mediator production, e.g., IL-1β, IL-6 and TNF-α. Knowing that mCRP is able to increase the production of these pro-inflammatory cytokines, it is feasible to hypothesize that this increase depends on an activation of these pathways.

NO and INOS in these immune-system cells (Ratajczak-Wrona et al., 2009). Moreover, a better understanding of the molecular mechanism involved in mCRP pro-inflammatory activity may pave the way for the identification a potential small molecular inhibitor capable of blocking, or at least reducing, this activity.

In this project also assessed the ability mCRP has to stimulate macrophage IFN-γ release. IFN-γ is another pro-inflammatory cytokine present in acute and chronic inflammation and is mainly released by T-cells and natural killer (NK) cells (Muhl and Pfeilschifter, 2003; Gomez et al., 2015; Kulkarni et al., 2016). Although previous studies have already reported that macrophages could release IFN-γ only when they had previously been co-stimulated by both IL-12 and IL-18 (Darwich et al., 2009), an ELISA assay was carried out to evaluate whether the mCRP pro-inflammatory proprieties could also be associated with an increase in this pro-inflammatory cytokine. However, as reported in the results section (**Fig. 4.10**) and confirmed in literature (Schoenborn and Wilson, 2007; Kulkarni et al., 2016) the macrophages did not release IFN-γ after mCRP (100 μg/mL) or LPS stimulation (10 ng/mL).

As aforementioned in the introduction paragraph of this chapter 4, a high FCγR expression was observed in oligodendrocytes, neurons, oligodendrocytes, astroglia, microglia, monocytes and macrophages (Okun et al., 2010). Macrophages have a high FCγRI (CD64), FCγRIIA (CD32a) and FCγRIII (CD16a) expression (Tanigaki et al., 2015). Bharadwaj et al. (1999) reported that nCRP was able to bind with the FCγRII receptors present in human U937 monocyte cell lines, whilst Eisenhardt et al. (2009b) reported that the nCRP-mCRP dissociation increased monocyte adhesion and ROS production, which was abolished when the FCγI (CD64), FCγII (CD32), FCγIII (CD16) receptors were blocked. This led to an assessment as to whether: (I) the FCγRs were able to mediate the mCRP pro-inflammatory activity; (II) whether this pro-inflammatory action is suppressed after the FCγRs are blocked.

The data obtained during this research showed that stimulation with FCγR antibodies: (I) did not induce any pro-inflammatory effects on U937 macrophages (**4.11** and **4.12**) (II); did not activate any signalling pathways potentially associated to a pro-inflammatory response (**4.13**); (III) did not reduce mCRP pro-inflammatory activity in U937monocyte-derived macrophages (**Fig 4.15**).

Despite the lack of direct and specific evidence, a possible explanation as to why FCγR antibodies were unable to block the mCRP pro-inflammatory activity may be found in literature. Indeed, Ji et al. (2009) confirmed that mCRP can trigger pro-inflammatory activity in human ECs when it binds with the lipid raft microdomain rather than to the FCγRs. Recently, Jia et al. (2018) reported that mCRP was capable of binding directly with the cholesterol binding sequence (CBS) in bone marrow-derived macrophages (BMDMs) and in peripheral blood mononuclear cells (PBMCs). Therefore, it is most likely that mCRP triggers its pro-inflammatory activity when it binds with the lipid raft microdomains or CBS present in the U937monocyte-derived macrophage cell line (**Fig 4.16**).

4.11 Key findings:

The key findings of the experiments described in this chapter are:

- U937 monocytes have a very high duplication capacity, despite their not being able to release pro-inflammatory TNF-α cytokines after LPS or mCRP stimulation. However, when U937 monocytes are PMA treated and successively stimulated with LPS they are able to differentiate and up-regulate the release of several cytokines, such as IL-1 β, TNF-α, IL-6 and IL-10.

- The results here reported confirm that mCRP interacts with macrophages and consequently leads to an up-regulated IL-1β gene expression and an increased concentration of pro-inflammatory cytokines (TNF-α and IL-6) when stimulated by mCRP (100 μg/mL) for 24 hours.

- The data herein reported corroborate that mCRP is not able to increase the release of IL-10 anti-inflammatory cytokines. A possible explanation may be that mCRP polarize the macrophage maturation towards the M1-like pro-inflammatory phenotype (Trial et al., 2016).

- The ELISA assay confirmed that mCRP stimulation increased TNF-α and IL-6 production but that anti-FCγR antibody stimulation does not reduce their production.

4.12 Summary.

Platelets can dissociate nCRP into mCRP during AD activation causing its accumulation in ischemic tissue (McFadyen et al., 2018). mCRP is capable of increasing in monocyte adhesion and the formation of ROS species when it binds with the FCγI (CD64), FCγIIa (CD32), FCγIII (CD16) receptors on ECs (Eisenhardt et al., 2009b). Knowing that the FC-γ receptors are also present in macrophages (Satpathy et al., 2012), the macrophages were stimulated with anti- FCγI (CD64) anti- FCγII (CD32) and anti- FCγIII (CD16) antibodies, to determine whether these receptors mediated the mCRP pro-inflammatory activity.

The experiments reported herein demonstrated that although mCRP have a strong pro-inflammatory activity, anti-FCγRs antibodies cannot reduce it. Therefore, mCRP may provoke pro-inflammatory activity by binding to other cellular portions (Ji et al., 2009; Jia et al., 2018). Further studies could hopefully clarify if there are any pathways that may be associated with pro-inflammatory activity, aimed at determining whether there are any antibodies and/ or SMIs capable of mitigating the mCRP pro-inflammatory action. This could be useful in order to block or at least limits the mCRP pro-inflammatory activity reported in several chronic inflammation condition (Schwedler et al., 2003; Eisenhardt et al., 2009b; Slevin et al., 2015).

CHAPTER 5 RESULT: Investigating the potential Acetylcholine anti-inflammatory activity on mCRP-stimulated monocyte differentiated macrophages.

CHAPTER 5: Investigating the potential Acetylcholine (ACh) and Nicotine anti-inflammatory activity on monocyte differentiated macrophages against mCRP.

As previously mentioned in chapter 1 paragraph 1.1.1, inflammation is a well regularised process useful to avoid substantial blood, fluid loss and potential infections (Weiss, 2008; Gurtner et al., 2008). Endothelial cells (ECs) increase the expression of both cell adhesion molecules (Martin, 1997) and chemokines (Deshmane et al., 2009) during the first phase of inflammation, recruiting neutrophils and monocytes (Rodero et al., 2014; Gonzalez et al., 2016). Once recruited, monocytes differentiated into naïve M0 macrophages which express specific surface makers (Murray and Wynn, 2011) that are capable of recognising Damage Associated Molecular Patterns (DAMPs) and Pathogen Associated Molecular Patterns (PAMPs) (Geissmann et al., 2010; Malissen et al., 2014; Orekhov et al., 2019). DAMPs, PAMPs and inflammatory stimuli bind with the M0 macrophages (Ramadan et al., 2017; Roh and Sohn, 2018), activating the MAPKs (JNK1/2, MAPKp38 and ERK 1/2), facilitating NF-KB phosphorylation which polarizes M0 macrophages into a pro-inflammatory M1 phenotype (Trial et al., 2016; Liu et al., 2017a; Liu et al., 2017c; Atri et al., 2018; Neamatallah, 2019). The polarization of M0 macrophages towards a pro-inflammatory M1 phenotype facilitates pro-inflammatory cytokine release (Scull et al., 2010; de Oliveira et al., 2017). When the pro-inflammatory cytokines bind with the the vagus nerve (VN), afferent fibres modulate the VN-efferent-fibre activity (Pavlov and Tracey, 2012). This neuromodulation leads to a cholinergic anti-inflammatory pathway (CAIP) activation, followed by an increase in non-neuronal Acetylcholine serum (Zhang et al., 2016; Han et al., 2017). ACh binds directly to the macrophages through the nicotinic acetylcholine receptors (nAChRs) (Rosas-Ballina et al., 2011). This binding reduces the NF-KB nuclear translocation **(Fig 5.1)** and limits the release of pro-inflammatory cytokines (Borovikova et al., 2000; Baez-Pagan et al., 2015).

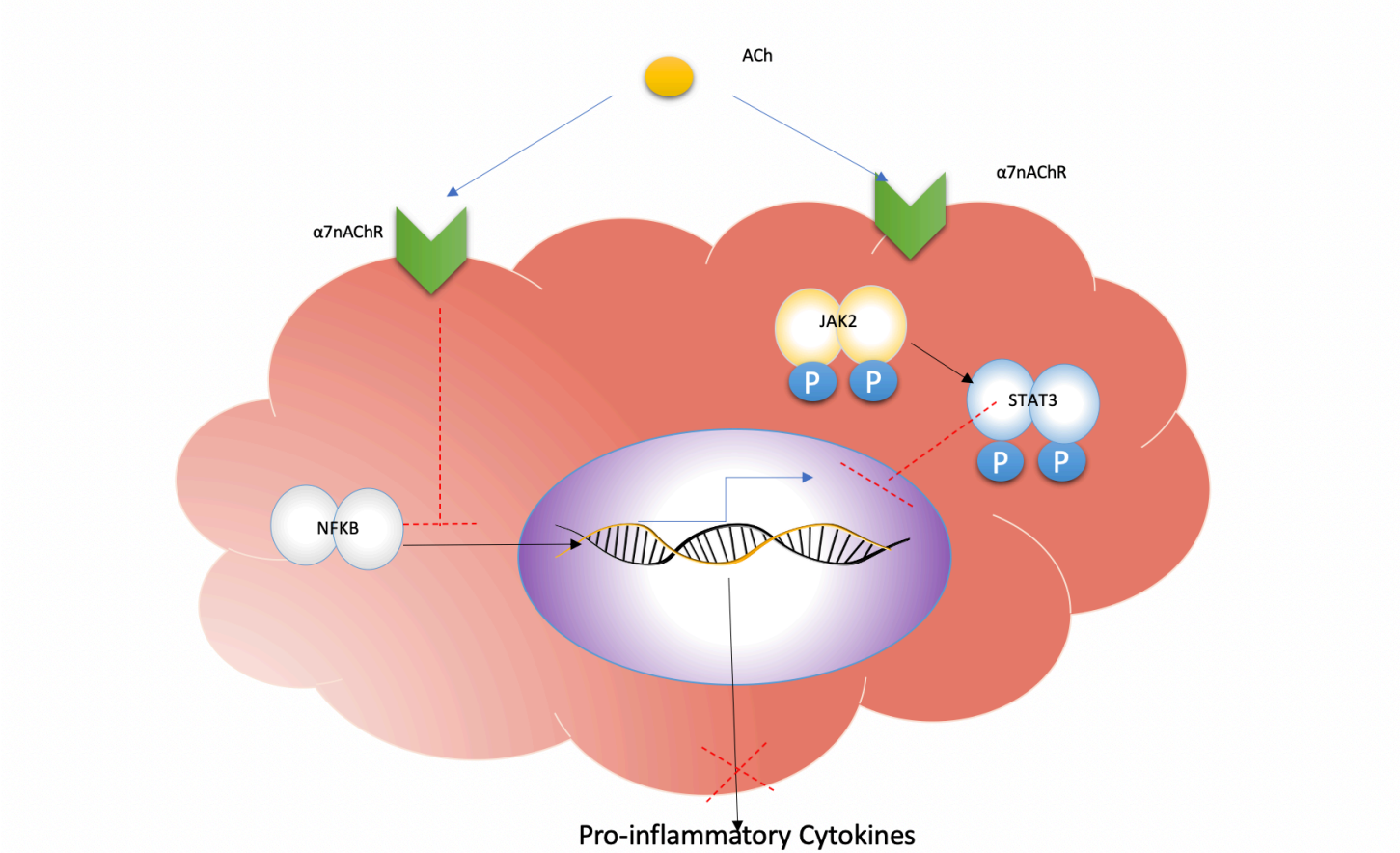


Fig 5.1. The protective effect of Acetylcholine (ACh). The efferent VN increases the activity of the spleen, liver and intestine, which, in turn increases ACh release. The anti-inflammatory activity is triggered when ACh binds with the α7 nicotinic acetylcholine receptor (α7nAChR) (expressed on macrophages or other immune cells), which regulates the NF-KB and STAT3 activity, reducing the pro-inflammatory cytokine release. Source: Adapted from Pavlov VA, Tracey KJ. The vagus nerve and the inflammatory reflex - Linking immunity and metabolism. Nature Reviews Endocrinology. 2012;8(12):743-54.

AChRs are cysteine-loop ligand-gated ion channel (cys-loopLGIC) receptors such us the γ-aminobutyric acid type A receptors (GABAA) and the serotonin 5-HT3 receptors (Cockcroft et al., 1990; Karlin, 2002; Le Novere et al., 2002). Several nAChR have been recognised in monocyte and macrophage (Galvis et al., 2006a; Chernyavsky et al., 2010). For example, Galvis et al. (2006b) reported that mice primary macrophages possess the α3, α4, α5, α7, α9, α10, β2, β3, and β4 subunit receptors and Chernyavsky et al. (2010) showed the presence of α1, α4, α5, α6, α7, α9, α10, β1, β2, β3, and β4 subunits on the U937 monocyte cell line. The specific activation of the α7nAChR subunit expressed on macrophages has been associated with strong anti-inflammatory activity (Nathan, 2002; Tracey, 2002; Wang et al., 2003; de Jonge et al., 2005; Saeed et al., 2005; Tracey, 2007). A recent study by Xu et al. (2019) reported on 35 preeclamptic pregnant women who had a downregulated α7nAChR in peripheral blood monocytes which led to a higher NF-KB transcription activity. This finding was also associated with an increase in the release of pro-inflammatory cytokines and a more severe clinical profile (Xu et al., 2019). More recently, the anti-inflammatory activity of α7nAChR have also been associated to an another α7nAChR agonist, i.e., Nicotine (Quik et al., 2015). As does ACh, this natural compound binds to α7nAChR, suppressing the NF-KB transcriptional activity which also reduces TNF-α pro-inflammaotry cytokine release (Yoshikawa et al., 2006b).

Although none of the SMIs under study blocked the dissociation from nCRP into mCRP, as reported in chapter 3 (**Fig. 3.5** and **Fig 3.6**)**,** the strong anti-inflammatory activity of ACh and Nicotine, prompted the evaluation of whether the use of ACh or Nicotine could be adopted as a therapeutic approach to block the detrimental pro-inflammatory effect of mCRP. Tests were also carried out during this project as to the possibility that ACh and Nicotine anti-inflammatory activities could potentially be mediated by the α7nAChR receptors.

Aim and objectives: To establish whether and how ACh and Nicotine could exercise anti-inflammatory activity against mCRP in monocyte-differentiated macrophages.

This was addressed by the following objectives:

- It was demonstrated in chapter 3 that neither ACh nor Nicotine were able to block nCRP-mCRP dissociation. However, recent studies have reported that both ACh and Nicotine treated macrophages release fewer pro-inflammatory cytokines (Borovikova et al., 2000; Tsoyi et al., 2011; Ren et al., 2017). As macrophages increase the release of pro-inflammatory cytokines when mCRP stimulated (chapter 4), the first objective was to determine whether ACh and/or Nicotine activity is able to block the mCRP pro-inflammatory process.

- As previously mentioned in chapter 1 paragraph 1.2.2.1, when monocytes differentiate into M0 resting macrophages (Ramadan et al., 2017; Roh and Sohn, 2018) they can polarise into an M1 pro-inflammatory phenotype (Atri et al., 2018; Neamatallah, 2019). mCRP might also be responsible for this process (Trial et al., 2016), increasing secretion levels of both TNF-α and IL-6 but not IL-10 anti-inflammatory cytokines (see chapter 4 paragraph 4.6). Pro-inflammatory mediator release in both monocytes and macrophages is downstream of mitogen-activated protein kinase (MAPKs) p38, Janus kinase/signal transducer and activator of transcription (JAK-STAT), extracellular signal-regulated kinases 1 and 2 (ERK1/2) and NF-KB (Moens et al., 2013; de Oliveira et al., 2017; Lim et al., 2018). However, whilst a study carried out in 2011 did not detect any critical intrinsic pathways activated by mCRP on THP-1 macrophages (Eisenhardt et al., 2011), further findings have reported that mCRP can increase the IL-6 concentration when both MAPKp38 and NF-KB signalling pathways are involved in ECs (Li et al., 2014). Thus, a second objective was to determine whether the MAPKs and NF-kB intrinsic pathway could be also activated in U937monocyte-differentiated macrophages by mCRP stimulation.

- It has been demonstrated that both ACh and Nicotine are capable of reducing the phosphorylation of MAPKs and of avoiding the nuclear translocation of NF-KB (Borovikova et al., 2000; Sun et al., 2013a; Yoshikawa et al., 2006b). That is why the third objective was to evaluate

whether when ACh and/or Nicotine reduce the MAPK and NF-KB intrinsic pathways, they also reduce the mCRP pro-inflammatory activity.

- The anti-inflammatory activity of ACh and Nicotine is triggered when they bind to α7nAChR (Sargent, 1993; Alkondon et al., 1997; Liu et al., 2012), α7nAChR is highly expressed in both monocyte and macrophage cells (Yoshikawa et al., 2006a; Hoover, 2017). When α7nAChR is activated it induces a strong anti-inflammatory activity (Sun et al., 2017; Ulleryd et al., 2019; Gowayed et al., 2019). This finding explains why this receptor is now indicated as a potential therapeutic drug for several medical conditions (Han et al., 2017; Xu et al., 2019). That is why the fourth objective was to assess whether ACh and Nicotine anti-inflammatory activity is mediated by α7nAChR. On the basis of the aforementioned, a specific α7nAChR antagonist was used to this aim.

5.1 Cytotoxicity evaluation of small molecule inhibitor (SMI) in U937-derived macrophages.

As aforementioned, ACh, and Nicotine have been shown to have anti-inflammatory properties (Han et al., 2017). However, investigation into their toxicity is a prerequisite before they may be proposed as a therapy against inflammation. Cell viability (Alamar Blue cell viability assay) and cell apoptosis assays (Annexin-V Fluorescein isothiocyanate apoptosis detection kit) were carried out after 24 hours of ACh and Nicotine stimulation (the best time-point for mCRP and LPS was obtained in experiment 4.5). On the basis of literature data, ACh was tested at several concentrations (from 0 to 250 μM) (Borovikova et al., 2000) and Nicotine (Mayer and Mayer, 2014) at its highest concentration of 150 ng/mL (**Fig 5.2**).

As reported in **Fig 5.2,** the Alamar Blue cell viability assay evidenced no cytotoxicity for ACh in the range of 0-250 μM. **Table 5.1** reports the percentage of viable cells for ACh. Also, Nicotine (between 0-150 ng/mL) did not show any cytotoxic effects. All the results are summarised in **Fig 5.2** and **Table 5.2**.

The cell viability test was followed by an apoptosis assay. ACh was tested at 100 μM and Nicotine at 150 ng/mL. These concentrations were selected on the basis of data from previous studies where they were used as inhibitors, in both macrophages and glial cells (Shytle et al., 2004; Park et al., 2007; Borovikova et al., 2000). No apoptotic effect was observed with 100 μM of ACh or 150 ng/mL of Nicotine (0.93 μM), as reported in **Fig 5.3** and **Table 5.3**. These results provided working concentrations for ACh (100 and 10 μM) and Nicotine (150 and 75 ng/mL) so as to evaluate SMI anti-inflammatory potential.

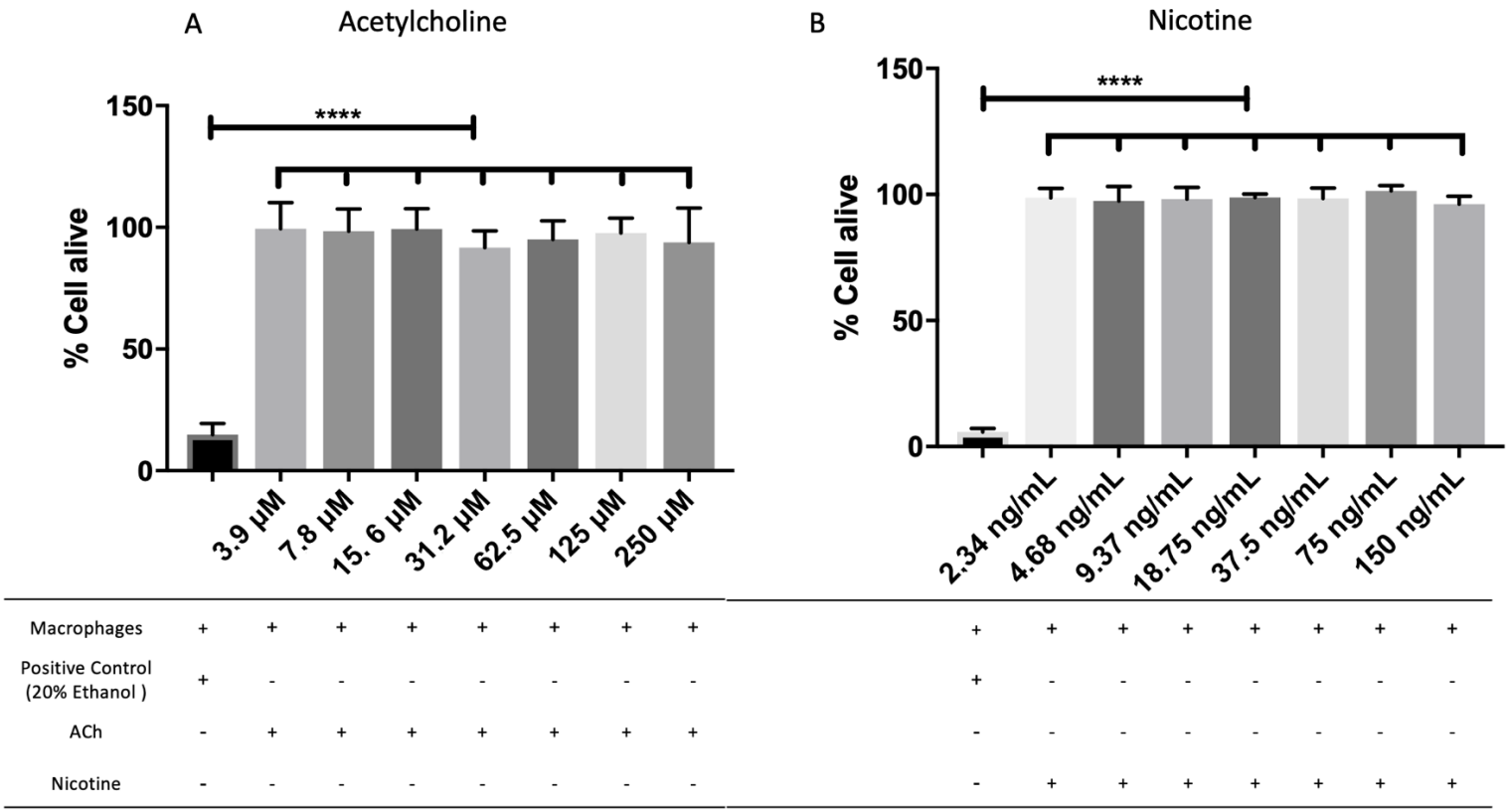


Fig 5.2. Cytotoxicity evaluation of small molecule inhibitor (SMI) in U937-derived macrophages. In this experiment a cytotoxic test was performed on ACh or Nicotine to evaluate any cytotoxic effects that might lead to cell death. After differentiation, monocyte differentiated macrophages were exposed to a media containing RPMI 1640 with L-glutamine (1640 medium Lonza) supplemented with 10% of FBS (F9665 Sigma®) and ACh (from 3.9 µM to 250 µM) or Nicotine (from 2.34 to 150 ng/mL) for 24 hours. Ethanol (20% total volume for 24 hours) was used as a positive control. Cell viability was evaluated by a CellTiter-Blue® Cell Viability Assay (Promega). The results are shown as the average ± SD. ****P-value < 0.0001. The ANOVA One-way and Tukey's post-hoc tests confirmed a statistically significant difference (P-value<0.0001) between the positive control (20% Ethanol) and all the groups stimulated with ACh (P-value<0.0001), or all the groups stimulated Nicotine (P-value<0.0001) (n=3).

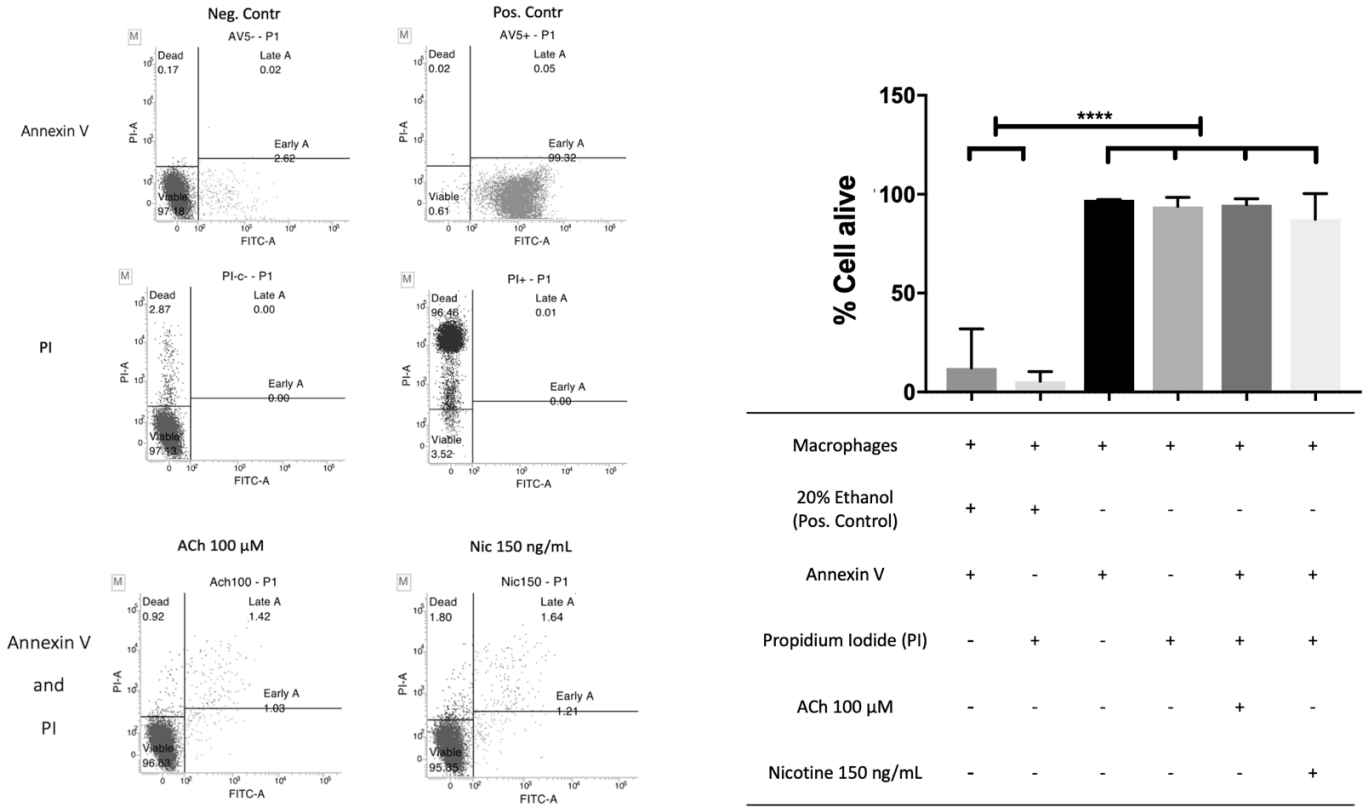


Fig 5.3. Cell apoptosis evaluation of small molecule inhibitor (SMI) U937-derived macrophages. After differentiation, monocyte-differentiated macrophages were cultured in media containing RPMI 1640 with L-glutamine (1640 medium Lonza) supplemented with 10% of FBS (F9665 Sigma®) and ACh (100 µM) or Nicotine (150 ng/mL) for 24 hours. Ethanol (20% total volume for 24 hours) was used as a positive control. Apoptosis was evaluated by Annexin-V Fluorescein isothiocyanate (FITC) apoptosis detection kit (Bioscience), according to the manufacturer's instructions. The results are shown as the average ± SD. ****P-value < 0.0001. The ANOVA one-way and Tukey's post-hoc tests confirmed a statistically significant difference (P-value<0.0001) between the untreated macrophage and the positive control (20% ethanol). The same results were confirmed by comparing the positive control with the group treated with SMIs (P-value<0.0001). No statistically significant difference (P-value=0.9678) was observed between the untreated macrophage and selected SMIs. The results confirmed that ACh (100 µM) and Nicotine 150 ng/mL (0.93 µM) did not lead to apoptotic body development (lower left quadrant of the AV/PI plot) in the U937 monocyte-differentiated macrophages (n=3).

ACh Alamar Blue Cell Viability Assay										
ACh Concentration		**Posi.Con**	**Neg. Con (0)**	**3.9 µM**	**7.8 µM**	**15.6 µM**	**31.2 µM**	**62.5 µM**	**125 µM**	**250 µM**
% Viable Cells		14.8%	100%	99.44%	98.45%	99.35%	91.70%	95.10%	97.65%	93.84%

Table 5.1. ACh Alamar Blue Cell Viability Assay. This table reports the percentage of viable cells after a cell viability assay was performed on U937 macrophages. Differently to the positive control (20% Ethanol), the SMI tested (ACh) was safe up to 250µM. (n=3).

Nicotine Alamar Blue Cell Viability Assay										
Nicotine Concentration		**Posi. Con**	**Neg. Con (0)**	**2.34 ng/ml**	**4.68 ng/m**	**9.37 ng/mL**	**18.75 ng/mL**	**37.5 ng/mL**	**75 ng/mL**	**150 ng/mL**
% ViableCells		5.83%	100%	98.68%	97.38%	98.22%	98.79%	98.44%	101.48%	96.24%

Table 5.2. Nicotine Alamar Blue Cell Viability Assay. This table reports the percentage of viable cells after a cell viability assay was performed on U937 macrophages. Differently to the positive control (20% ethanol), the SMI tested (Nicotine) was safe up to 150 (0.93 µM) ng/mL. (n=3).

SMI Cell Apoptosis Assay							
Cell apoptosis assay		**Neg Annexin V**	**Neg PI**	**Pos Annexin V**	**Pos PI**	**ACh 100 µM**	**Nic 150 ng/mL**
% Viable Cells		97.15%	93.81%	12.18%	5.38 %	94.67%	87,.29%

Table 5.3. SMI Cell Apoptosis Assay. A test was performed to assess whether the SMIs showed any cytotoxic effects that could lead to cell death. Cell viability was unaffected by ACh at 100 µM and Nicotine at 150 ng/mL. Only one concentration for each compound was used in this cell apoptosis assay. The percentage of viable cells expressed is the average of three different experiments. (n=3).

5.2 The effect SMIs have on inflammation in U937 macrophages.

The previous experiments allowed for the determination of the best working SMI concentrations to use without triggering cytotoxic effects that might lead to cell death. Therefore, ACh was used at concentrations of 100 μM and 10 μM and Nicotine at 150 and 75 ng/mL (0.93 and 0.46 μM respectively). These concentrations for the same reason described in paragraph 5.1 (Borovikova et al., 2000; Shytle et al., 2004; Park et al., 2007). The next step was to determine that selected SMIs did not effectively induce any pro-inflammatory effects in U937 macrophages. In the experiments described as follows the pro-inflammatory effects of the anti-mCRP 3H12 antibody was also investigated. LPS at 10 ng/mL was used as a positive control, as previously optimized in chapter 4 paragraph 4.4. Macrophages were exposed to SMIs in the aforementioned concentration for 24 hours (see experiment paragraph 5.1). TNF-α, IL-6, or IL-10 were selected as targets as described in the experiments in chapter 4, paragraph 4.6.

As reported in (**Fig 5.4** and **Fig 5.5**) enzyme-linked immunosorbent assays (ELISA) were carried and demonstrated that SMIs alone did not increase the macrophage cytokine release. An assessment and comparison (**Fig 5.4**) was made of the TNF-α protein concentrations for the untreated macrophage group, the positive control group and the group stimulated with ACh (100 μM and 10 μM) or Nicotine (150 ng/mL and 75 ng/mL) at 24 hours. No statistically significant difference was observed between the untreated group and the groups stimulated with ACh at 100 μM or 10 μM and Nicotine at 150 ng/mL and 75 ng/mL. Whereas, there was a statically significant difference between the untreated macrophage group vs the positive control group, as was observed in the positive control vs the other the groups stimulated with ACh at at 100 μM or 10 μM and Nicotine at 150 ng/mL and 75 ng/mL. Furthermore, a statistical difference was confirmed between the untreated macrophage group and the group treated with the 3H12 anti-mCRP antibody.

As shown in **Fig 5.4,** assessment and comparison was made of the IL-6 protein concentrations in the untreated macrophage group, the positive control and

the group stimulated with ACh (100 μM and 10 μM) or Nicotine (150 ng/mL and 75 ng/mL) at 24 hours. No statistically significant difference was observed between the untreated macrophage group the ACh and Nicotine groups. Whilst there was a significant difference between the untreated macrophage group and the positive control group, as well as the positive control group and the other the groups stimulated with ACh at ACh at at 100 μM or 10 μM and Nicotine at 150 ng/mL and 75 ng/mL. Furthermore, concentrations also differed between the untreated macrophage group and the group treated with the 3H12 anti-mCRP antibody.

As shown in **Fig 5.5** assessment and comparison was made of the IL-10 protein concentrations in the untreated macrophage group, the positive control group and the groups stimulated with SMIs at 24 hours. No statistically significant difference was observed between the untreated macrophage group and the ACh and Nicotine groups, nor was there a statistically significant difference between the ACh and Nicotine groups.

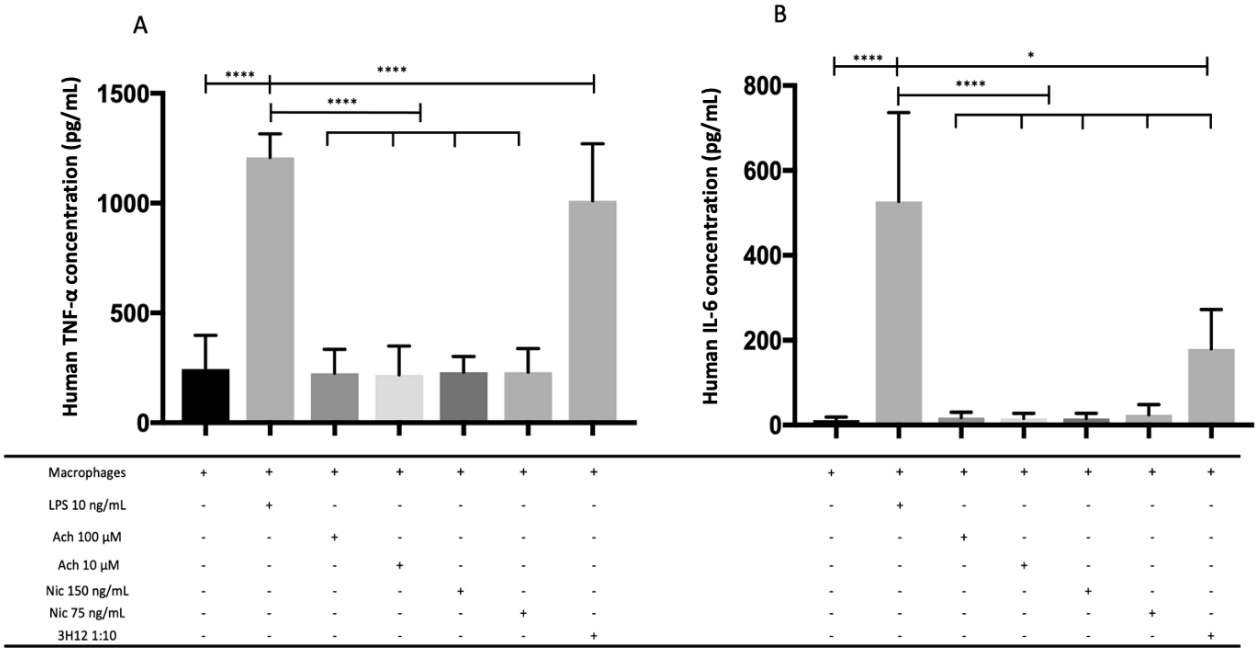


Fig 5.4. SMIs did not induce pro-inflammatory effects on U937 macrophages. After differentiation (PMA 50 ng/mL for 72 hours), the macrophages were treated with LPS at 10 ng/mL (positive control) or ACh (100 and 10 µM) or Nicotine (150 and 75 ng/mL) or anti-3H12 antibody (1/10 total volume), for 24 hours. The TNF-α (A)- and IL-6 (B) production was quantified by the ELISA assay (R&D System). The results are shown as the average ± SD. ****P-value <0.0001. *P-value<0.001. There was an increase of the TNF-α (A) concentration and IL-6 (B) pro-inflammatory cytokines in the positive control group. The absence of statistical differences (P-value=0.3157) between the untreated macrophage group and SMIs groups (A) was confirmed by the One-way ANOVA test, followed by Tukey's post-doc test, with a 99.9% CI and a 0.01% α error. A statistically significant difference was observed when the positive control and the SMI groups were compared (P-value<0.0001) and when the untreated macrophage group (P-value=0.0230) and the group treated with the 3H12 anti-mCRP antibody (244.6 vs 1011 pg/mL respectively), were compared. A similar result was also confirmed with IL-6. No statistical differences (P-value> 0.9999) between the untreated macrophage group and the SMIs groups (B) were observed, as confirmed by the One-way ANOVA test, followed by Tukey's post-doc test, with a 99.9% CI and α error fixed at 0.01%. The only statically significant difference was observed (P-value<0.001) when the untreated macrophage group and the anti-3H12 antibody group (9.797 vs 172.5 pg/mL respectively) were compared. (n=3).

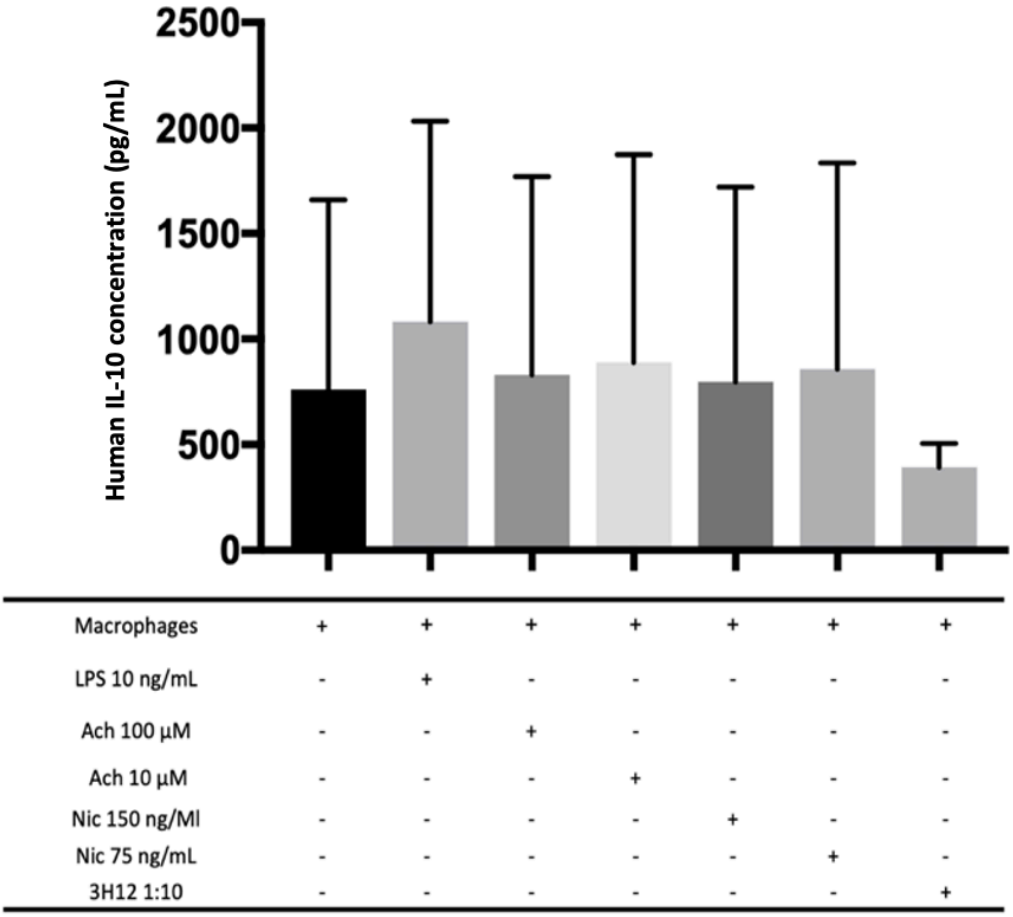


Fig 5.5. SMIs did not increase the release of IL-10 anti-inflammatory cytokine. After differentiation (PMA 50 ng/mL for 72 hours), the macrophages were treated with LPS at 10 ng/mL (positive control), ACh (100 and 10 µM), Nicotine (150 and 75 ng/mL) or anti-3H12 antibody (1/10 total volume), for 24 hours. The production of IL-10 anti-inflammatory cytokines was quantified by ELISA assay (R&D System). The results are shown as the average ± SD. ****P-value <0.0001. *P-value<0.001. The One-way ANOVA test, followed by Tukey's post-doc test, with a 99.9% CI and a 0.01% α error, confirmed there were no statistically significant differences (P-value=0.3157) amongst the groups (P-value=0.7063) (n=3).

5.3 Testing the anti-inflammatory activity of SMIs on LPS-stimulated macrophages.

After having demonstrated that SMIs did not induce pro-inflammatory activity on macrophages, the general anti-inflammatory properties of the SMIs (ACh and Nicotine) were evaluated (Han et al., 2017; Yamada and Ichinose, 2018). The SMIs were tested against the positive control (LPS at 10 ng/mL) for 24 hours. TNF-α, IL-6 and IL-10 were selected as markers as reported in chapter 4, paragraph 4.6. The macrophages were pre-treated with SMIs for 2 hours as this pre-treatment time point had been demonstrated to be the best time-point (experiment 5.4).

There was a statistically significant increase in the IL-6, IL-10 and TNF-α level in macrophages following stimulation with LPS at a concentration of 10 ng/mL.

As reported in **Fig. 5.6** and **Fig. 5.7**, the trend here reported showed that the use of SMIs did not reduce this release. SMIs were not found to be characterised by any general anti-inflammatory effects.

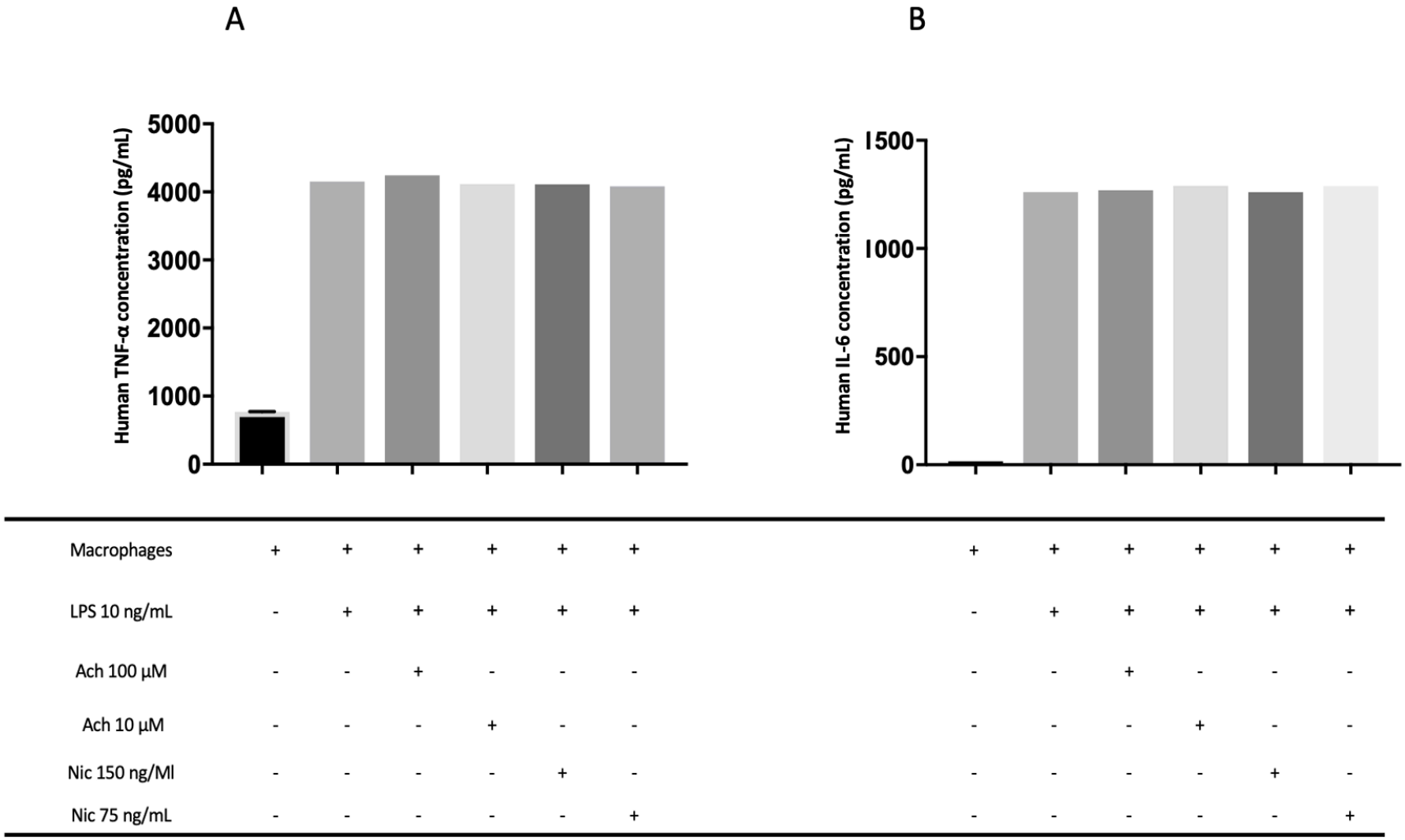


Fig 5.6. None of the SMIs showed anti-inflammatory properties. Monocyte differentiated macrophages were pre-treated with ACh (100 and 10 µM) or Nicotine (15 and 75 ng/mL) for 2 hours and then stimulated by LPS (10 ng/mL) for 24 hours. LPS alone (10 ng/mL) was used as a positive control, for 24 hours. The pro-inflammatory cytokines TNF-α and IL-6 were assessed by ELISA assay (R&D System). Stimulation by LPS, at 10 ng/mL for 24 hours led to a substantially increased production of both TNF-α (A) and IL-6 (B) in the macrophages and this cytokine release remained extremely high even after the use of SMIs (n=1 2 technical replicates).

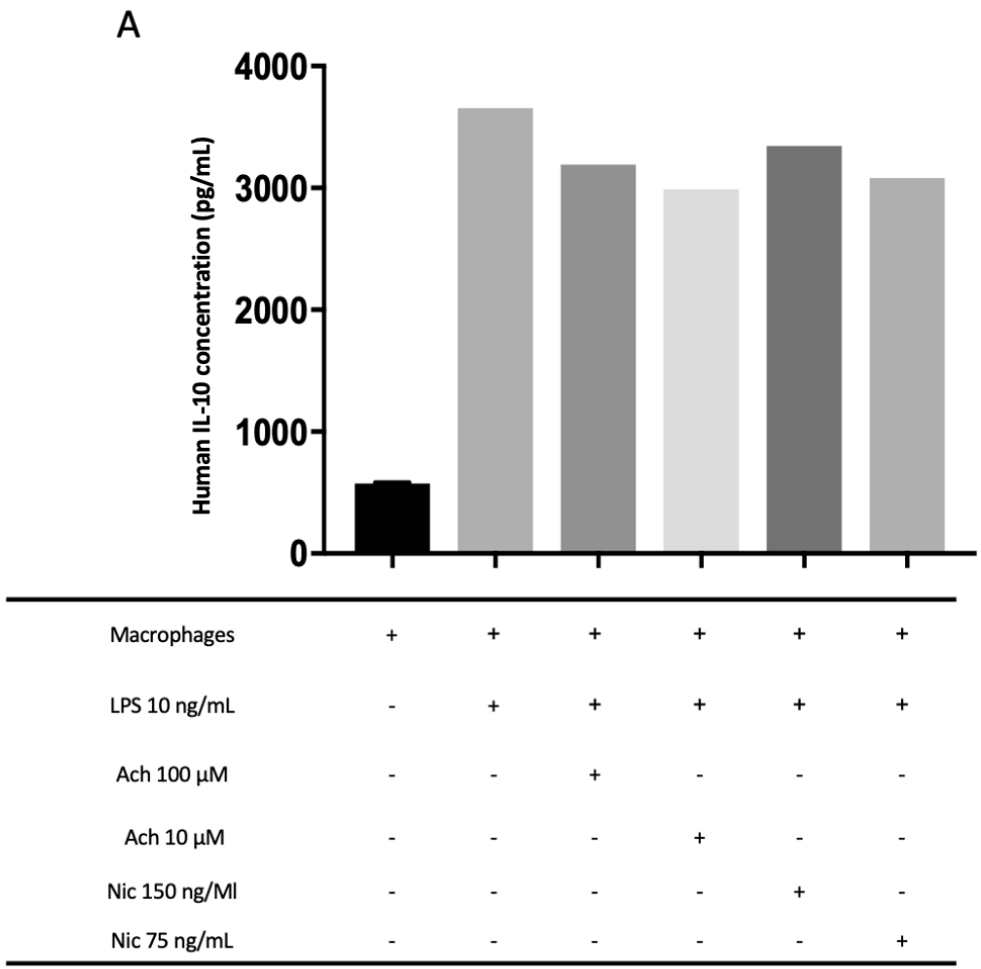


Fig 5.7. SMIs did not reduce the IL-10 anti-inflammatory cytokines release. The macrophages were pre-treated with ACh (100 and 10 µM) or Nicotine (150 and 75 ng/mL) for 2 hours and then stimulated by LPS (10 ng/mL) for 24 hours. Only LPS (10 ng/mL) was used for 24 hours as a positive control. The anti-inflammatory cytokines IL-10 was assessed by ELISA assay (R&D System). Stimulation by LPS, at 10 ng/mL for 24 hours, led to a substantially increased production of IL-10 in macrophages and this cytokine release remained extremely high even when the macrophages were pre-stimulated by SMIs (n=1 2 technical replicates).

5.4 Evaluation of the incubation time-point for SMIs, aimed at the inhibition of mCRP pro-inflammatory activity.

After observing that the SMIs did not induce pro or anti-inflammatory effects on LPS-stimulated macrophages, assessment was made as to whether SMIs could limit or block mCRP inflammatory activity. To this aim, it was necessary to optimise the SMIs in terms of time points. The same SMI concentrations as that used in experiment 5.2. were adopted. The mCRP concentrations and time-points were the same as those in experiment 4.6. TNF-α, was selected based on the same concepts expressed in chapter 4, paragraph 4.6 and were used to evaluate the SMI anti-inflammatory proprieties. The TNF-α, cytokine concentration was quantified by the ELISA assay.

As shown in **Fig 5.8**, the trend showed a statistically significant difference between the TNF-α expression level in the untreated macrophage group and macrophages stimulated with mCRP (18.71 vs 1203 pg/mL). It was observed that a four-hour pre-treatment with Nicotine 150 ng/mL (0.93 μM) or 3H12 was able to reduce TNF-α release (1203 vs 480 pg/mL and 1203 vs 943.3 pg/mL respectively).

A comparison between ACh and mCRP groups indicated that the levels of TNF-α in U937-monocyte differentiated macrophages pre-stimulate for two hours with ACh both at 100 and 10 μM was lower than that in the mCRP group alone (1203 vs 639 pg/mL and 1203 vs 651.1 pg/mL respectively). Similarly, all groups that had been pre-treated for 2 hours with Nicotine (150 ng/mL) and antimCRP-3H12 antibody (1:10) had a reduction in mCRP pro-inflammatory activity.

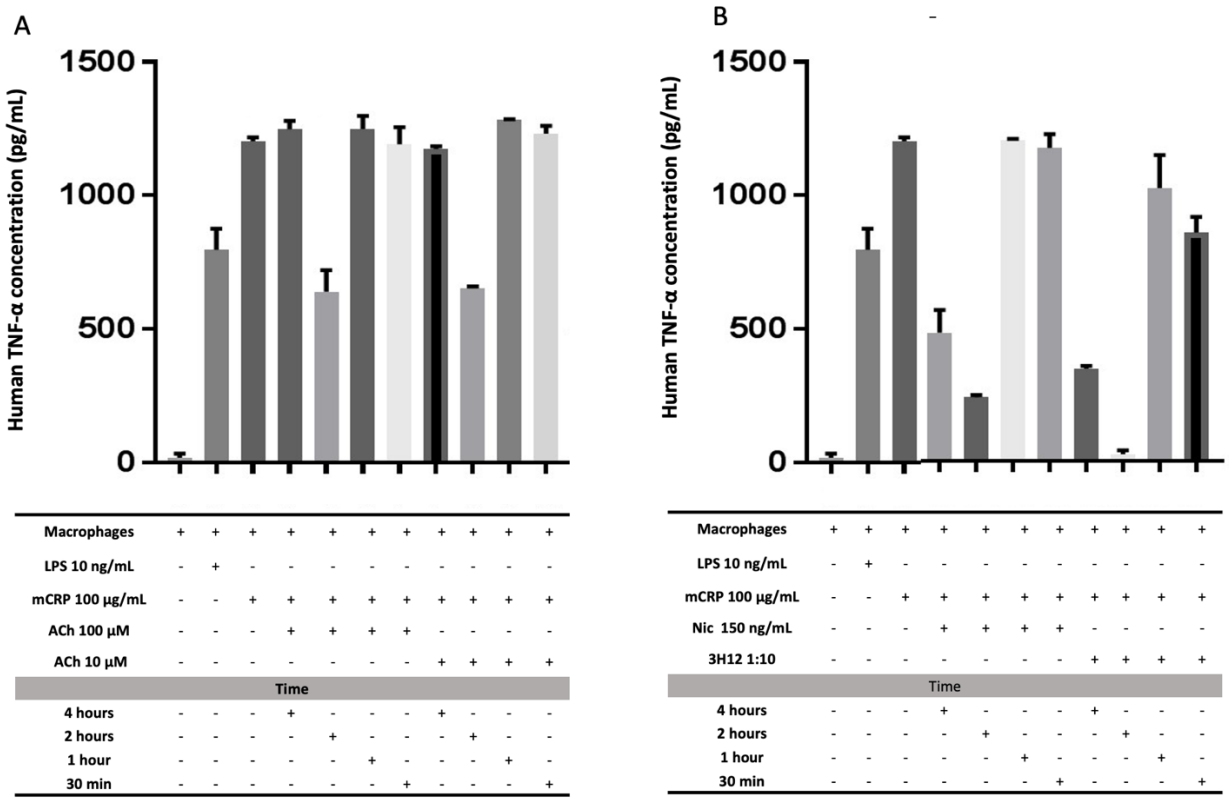


Fig 5.8. Time-point evaluation activity for SMIs, aimed at the inhibition of mCRP pro-inflammatory activity. After differentiation (PMA exposure 50 ng/mL for 72 hours) monocyte differentiated macrophages were pre-treated with ACh (100 and 10 µM), Nicotine (150 and 75 ng/mL) or antimCRP-3H12 antibody (1:10) for 30 minutes 1, 2 and 4 hours respectively, followed by stimulated with mCRP (100 µg/mL) for 24 hours. LPS (10 ng/mL for 24 hours) was used as a positive control. The pro-inflammatory cytokine TNF-α was estimated by ELISA assay (R&D System). Fig (A-B) mCRP stimulation at 100 µg/mL increased TNF-α production. The trend showed that pre-stimulation with ACh (100 and 10 µM) led to anti-inflammatory activity. B) The trend showed that pre-stimulation with Nicotine (150 and 75 ng/mL) or antimCRP-3H12 antibody (1:10) led to anti-inflammatory activity. Two hours was the optimum time-point to reduce the TNF-α release for all conditions. At 4 hours the SMI inhibitors began to lose their efficacy. (n=1 2 technical replicates).

5.5 Studying the SMI-ACh anti-inflammatory effect against mCRP.

Previous research demonstrated that, after binding with macrophages, both ACh and Nicotine have strong anti-inflammatory activity (Boras et al., 2014; Han et al., 2017). This experiment assessed the anti-inflammatory effect of SMIs against mCRP, i.e., its ability to reduce pro-inflammatory effects. The U937-derived macrophages were incubated for 2 hours (see 5.4 for time point experiment) with ACh (100 and 10 μM), Nicotine (150 and 75 ng/mL) or the antimCRP-3H12 antibody (1:10). The concentrations were the same as those reported in 5.2 and 5.4 for all the SMIs. After this initial pre-treatment, the macrophages were exposed to 100 μg/mL mCRP for 24 hours (see experiment 4.6). TNF-α, IL-6 and IL-10 were selected, according to the results obtained in chapter 4 paragraph 4.6, as cytokines of interest. The cytokine concentrations were quantified by ELISA assay.

ELISA assay was carried out in order to evaluate the TNF-α releases after mCRP stimulation. It was observed that mCRP increased the release of TNF-α (P-value<0.0001). The mCRP pro-inflammatory effect was comparable to that of the positive control group (LPS 10 ng/mL). No statistically significant differences were observed between the positive control group and the one stimulated by mCRP (**Fig 5.9**). The small molecule (ACh and Nicotine) and anti-mCRP3H12 antibody anti-inflammatory activity were also investigated. Pre-treatment with ACh at 100 μM (P-value<0.0001) and 10 μM (P-value=0.0110) significantly reduced TNF-α release. No statistically significant differences were observed between the mCRP stimulated group and all other groups that had been pre-stimulated with Nicotine or the 3H12 antibody.

The ELISA assay quantified the IL-6 releases after mCRP stimulation. The mCRP stimulation increased the release of the IL-6 pro-inflammatory cytokine (P-value<0.0001). The IL-6 cytokine release is similar to that observed in the positive control group (LPS 10 ng/mL). There were no statistically significant differences between the positive control group and the one stimulates by mCRP. The SMIs ACh, Nicotine, and anti-mCRP3H12 antibody anti-inflammatory activity was also assessed. Only the pre-treatment with ACh at

100 μM was capable of reducing IL-6 production (P-value<0.0001). No statistically significant differences were observed in any of the other groups stimulated by ACh (10 μM), Nicotine (P-value 0.9810) or the 3H12 antibody (P-value 0.9810).

The ELISA assay quantified the IL-10 releases after mCRP stimulation. As shown **Fig 5.10**, contrary to mCRP, LPS stimulation increased the anti-inflammatory cytokine IL-10 secretion significantly (P-value 0.0305). There were no statistically significant differences (P-value 0.9830) in any of the other groups that had previously been stimulated by SMIs (ACh or Nicotine) or the 3H12 antibody. **Fig 5.10** reports the results.

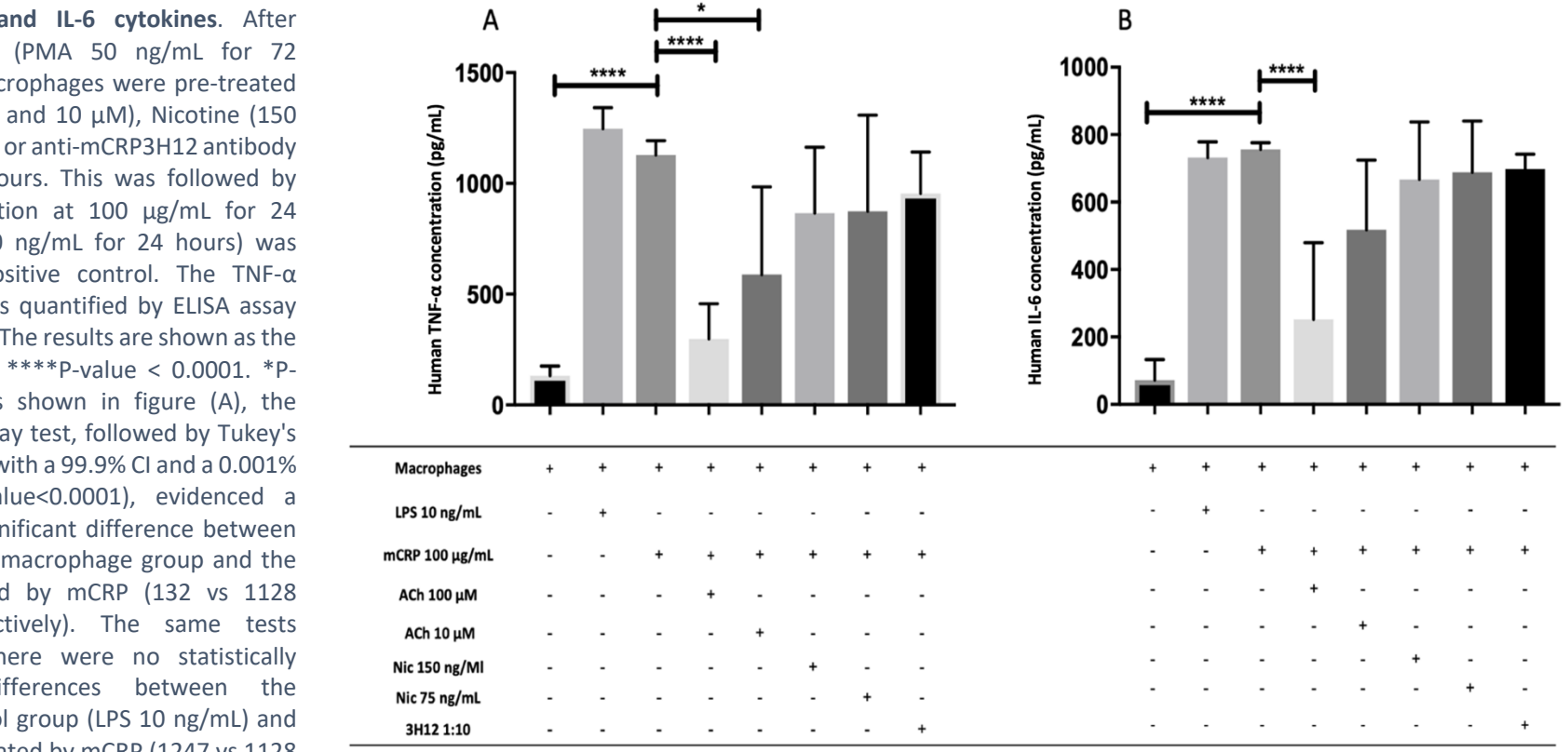
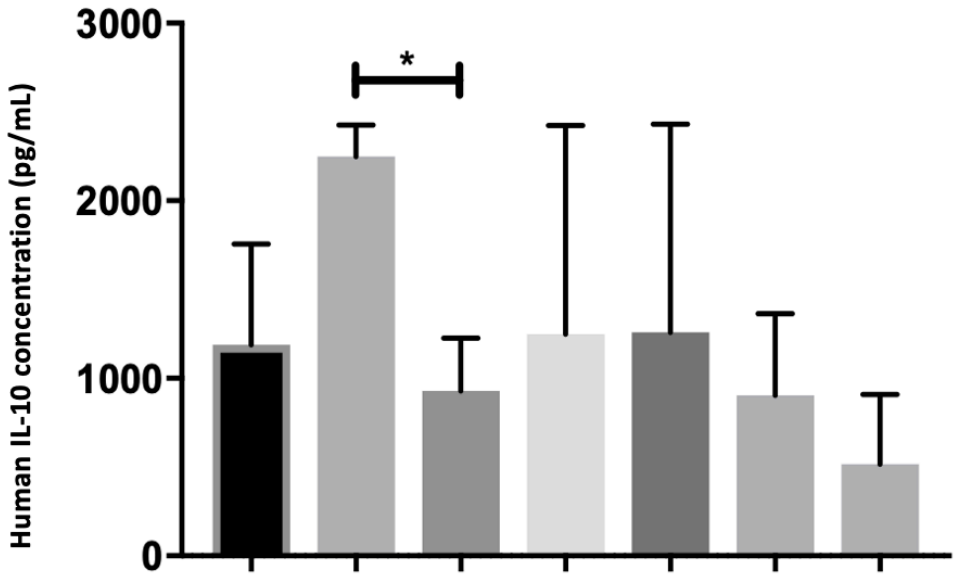


Fig 5.9. ACh reduced the concentration of both TNF-α and IL-6 cytokines. After differentiation (PMA 50 ng/mL for 72 hours) the macrophages were pre-treated with ACh (100 and 10 µM), Nicotine (150 and 75 ng/mL), or anti-mCRP3H12 antibody (1:10) for 2 hours. This was followed by mCRP stimulation at 100 µg/mL for 24 hours. LPS (10 ng/mL for 24 hours) was used as a positive control. The TNF-α production was quantified by ELISA assay (R&D System). The results are shown as the average ± SD. ****P-value < 0.0001. *P-value<0.05. As shown in figure (A), the ANOVA One-way test, followed by Tukey's post-hoc test, with a 99.9% CI and a 0.001% α error (P-value<0.0001), evidenced a statistically significant difference between the untreated macrophage group and the one stimulated by mCRP (132 vs 1128 pg/mL respectively). The same tests determined there were no statistically significant differences between the positive control group (LPS 10 ng/mL) and the one stimulated by mCRP (1247 vs 1128 pg/mL respectively). The ANOVA One-way test also showed that there were statically significant differences (P-value<0.0001) in the TNF-α expression level between the groups where the macrophages had been stimulated by mCRP alone and those where mCRP had previously been incubated with ACh at 100 µM (1128 vs 298.4 pg/mL respectively) and 10 µM (1128 vs 581.7 pg/mL respectively). The TNF-α reduction in macrophages previously treated with SMI ACh (both 100 and 10 µM) was so significant that the ANOVA One-way did not report any statistical differences between the untreated macrophage group and the ACh stimulated groups (P-value 0.0573). As reported in figure (B), the ANOVA One-way test, followed by Tukey's post-hoc test, with a 99.9% CI and a 0.001% α error, evidence a statistically significant difference (P-value <0.0001) between the untreated macrophage group and the mCRP stimulated macrophages (72.32 vs 756.6 pg/mL respectively). The same tests evidenced no statistically significant differences (P-value >0.9999) between the positive control group and the mCRP stimulated macrophages (732.4 vs 756.6 pg/mL respectively). However, significant differences (P-value<0.0001) were observed between the mCRP-stimulated macrophages and the groups where the mCRP had previously been incubated with ACh at 100 µM (756.6 vs 251.7 pg/mL respectively). This reduction was so significant that the ANOVA One-way test did not report any statistically significant differences between the untreated macrophage group and the group pre-stimulated with ACh 100 µM (P-value =0.4548) (n=3).



Macrophages	+	+	+	+	+	+	+	+
LPS 10 ng/mL	-	+	-	-	-	-	-	-
mCRP 100 µg/mL	-	-	+	+	+	+	+	+
ACh 100 µM	-	-	-	+	-	-	-	-
ACh 10 µM	-	-	-	-	+	-	-	-
Nic 150 ng/Ml	-	-	-	-	-	+	-	-
Nic 75 ng/mL	-	-	-	-	-	-	+	-
3H12 1:10	-	-	-	-	-	-	-	+

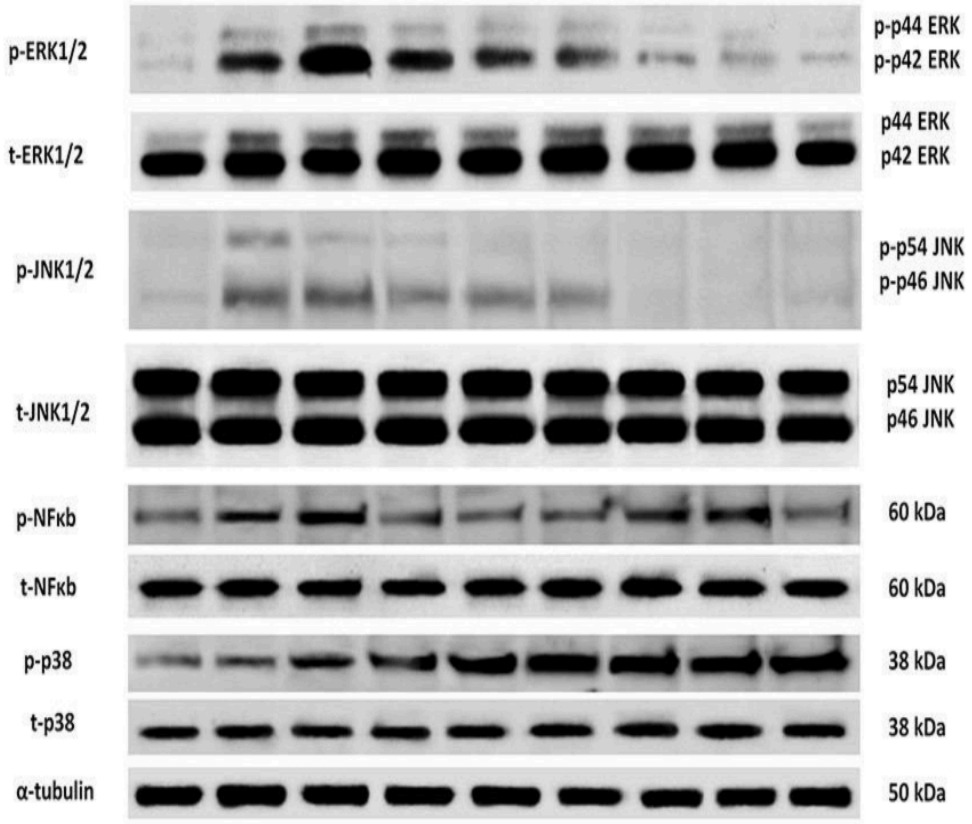
Fig 5.10. ACh did not affects the release of IL-10 anti-inflammatory cytokine. The macrophages were pre-treated with ACh (100 and 10 µM), Nicotine (150 and 75 ng/mL), or anti-mCRP3H12 antibody (1:10) for 2 hours. This was followed by mCRP stimulation, at 100 µg/mL, for 24 hours. LPS (10 ng/mL for 24 hours) was used as a positive control. The production of IL-10 was quantified by ELISA assay (R&D System). The results are shown as the average ± SD. ****P-value<0.0001. *P-value<0.05. The ANOVA One-way test, followed by Tukey's post-hoc test, with a 99.9% CI and a 0.001% α error, evidenced no statistically significant difference (P-value= 0.9994) between the untreated macrophage group and the mCRP stimulated groups (1190 ± 231.2 vs 929 ± 120.9 pg/mL respectively). The ANOVA One-way test also confirmed a statistically significant difference (P-value= 0.0305) between the positive control group and the mCRP stimulated groups (2249 vs 929 pg/mL respectively). No statistical differences (P-value=0.9830) were observed in any of the other groups that had previously been stimulated by SMIs (ACh or Nicotine) or the 3H12 antibody (n=3).

5.6 The role of both mitogen-activated protein kinases (MAPKs) and NF-KB pathways in response to mCRP pro-inflammatory activity.

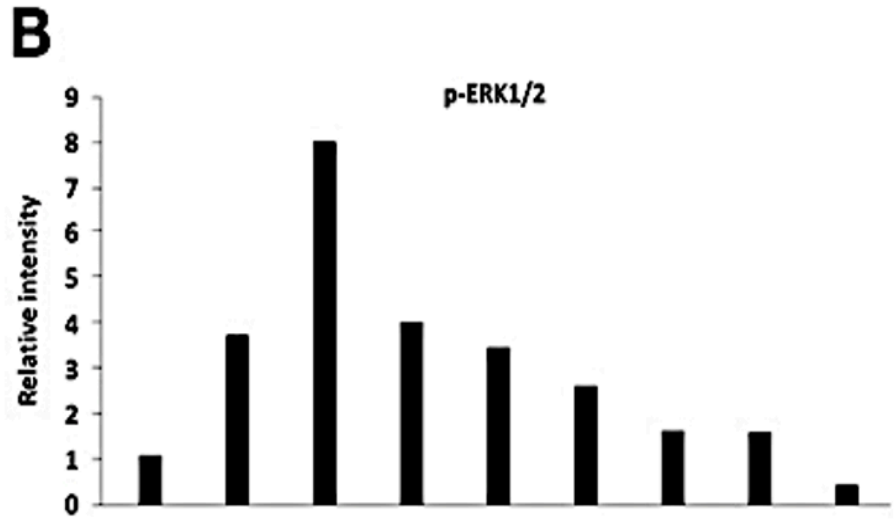
After having determined that mCRP has a pro-inflammatory activity, the possible signalling pathways were investigated. As previously reported in chapter 1 paragraph 1.1.9, literature has reported that the activation of MAPKp38, JNK and ERK1/2 facilitates pro-inflammatory cytokine release (Kyriakis and Avruch, 2001). It was reported by de Oliveira et al. (2017) that when macrophages were treated by LPS, they up-regulated the MAPKp38/JNK/NF-KB intrinsic pathways through an increase in pro-inflammatory cytokines, such as IL-1β and TNF-α levels. Lim et al. (2018) reported that LPS-induced phosphorylation of both ERK and JNK but not MAPKp38 in BV-2 Microglia. It is known that mCRP pro-inflammatory activity in both leukocytes and ECs is triggered when MAPKs or NF-KB are activated (Tarek et al., 2002; Tarek et al., 2004; Tarek et al., 2006; Turu et al., 2008). However, to the best of my knowledge, no studies have been able to detect any critical intrinsic pathways activated by mCRP on macrophages (Eisenhardt et al., 2011). Thus, a western blot assay was carried out to study whether activation of MAPKp38, JNK, ERK 1/2 and NF-KB through their phosphorylation, were linked to mCRP pro-inflammatory activity in monocyte U937 differentiated macrophages. Next, ACh and Nicotine were also tested before incubation with mCRP to assess whether they were capable of reducing MAPKp38, JNK, ERK 1/2 and NF-KB phosphorylation in agreement with data from international literature (Yoshikawa et al., 2006b; Sun et al., 2013a; Lim et al., 2018).

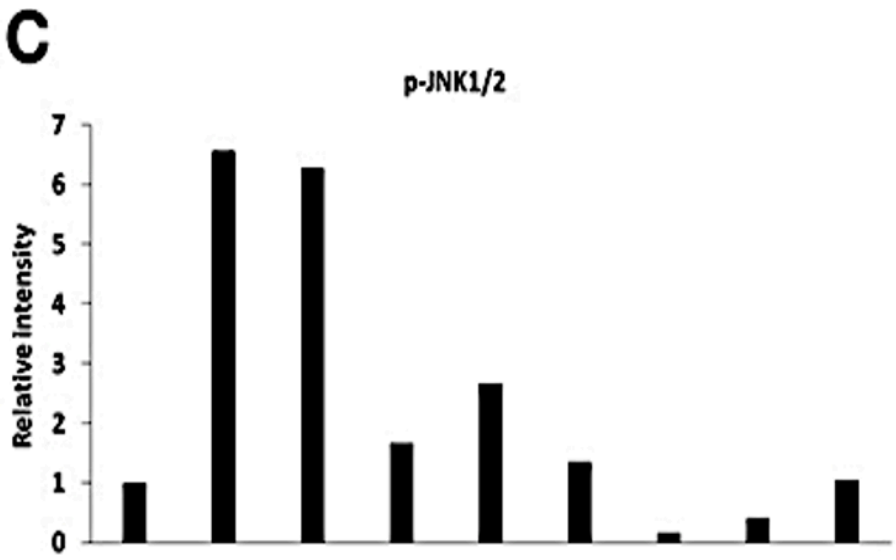
The results reported in **Fig 5.11** showed that LPS, at 10 ng/mL and the use of mCRP alone, at 100 µg/mL, increased the phosphorylation level of ERK 1/2, JNK 1/2, MAPKp38 and NF-KB. These results (**Fig 5.11**) also confirmed that both ACh, at 100 and 10 µM and Nicotine alone at 150 ng/mL, do not increase the phosphorylation of ERK 1/2 and JNK 1/2 but they do increase the release of MAPKp38. The NF-KB phosphorylation was also increased when the macrophages were exposed to ACh (100 and 10 µM) alone. The resulting data also showed that when macrophages were pre-treated with ACh (100 and 10

μM) or Nicotine (150 ng/mL) both the SMIs were also able to reduce the ERK 1/2, JNK 1/2 and NF-KB phosphorylation induced by mCRP (100 μg/mL). However, neither ACh nor Nicotine were able to reduce the mCRP-MAPKp38 phosphorylation.



Macrophages	+	+	+	+	+	+	+	+	+
LPS 10 ng/mL	-	+	-	-	-	-	-	-	-
mCRP 100 μg/mL	-	-	+	+	+	+	-	-	-
ACh 100 μM	-	-	-	+	-	-	+	-	-
Ach 10 μM	-	-	-	-	+	-	-	+	-
Nic 150 ng/mL (0.93 μM)	-	-	-	-	-	+	-	-	+





Macrophages	+	+	+	+	+	+	+	+	+
LPS 10 ng/mL	-	+	-	-	-	-	-	-	-
mCRP 100 µg/mL	-	-	+	+	+	+	-	-	-
ACh 100 µM	-	-	-	+	-	-	+	-	-
ACh 10 µM	-	-	-	-	+	-	-	+	-
Nic 150 ng/mL (0.93 µM)	-	-	-	-	-	+	-	-	+

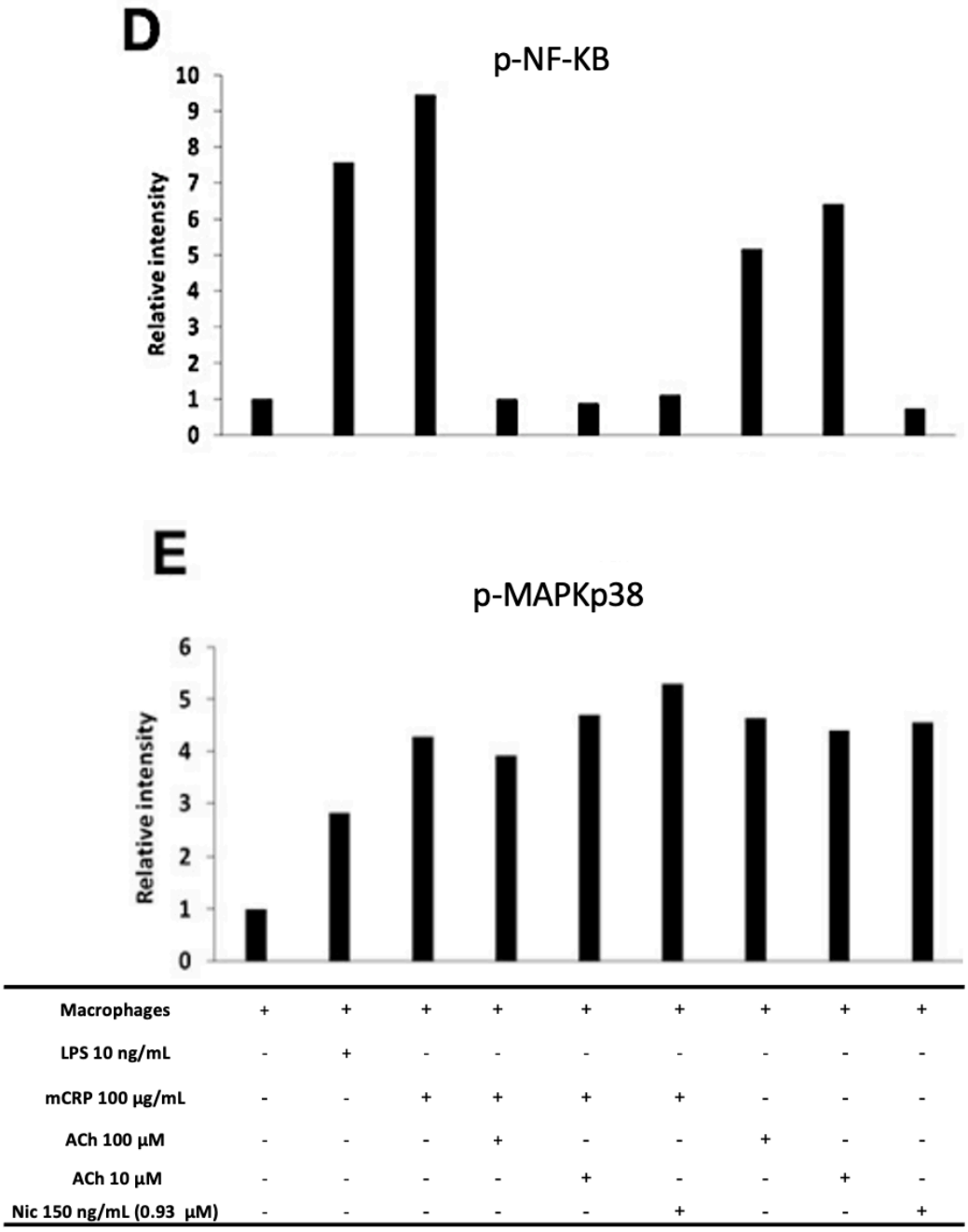


Fig 5.11. The role of both MAPKs and NF-KB pathways in response to mCRP pro-inflammatory activity. Following differentiation (PMA 50 ng/mL for 72 hours), monocyte-differentiate macrophages were stimulated by ACh (100 and 10 µM), or Nicotine (150 ng/mL) whether or not mCRP (100 µg/mL) was present for eight minutes. LPS (10 ng/mL) for 8 minutes was used as a positive control. After disposal of the medium, the macrophages were washed twice with PBS, followed by addition of 250 µl of lysis buffer containing a protease inhibitor and phosphatase inhibitor cocktails. Lane 1, untreated macrophage; Lane 2, LPS (10 ng/mL); Lane 3 mCRP (100 µg/mL); Lane 4 ACh (100 µM) + mCRP (100 µg/mL); Lane 5 ACh (10 µM) + mCRP (100 µg/mL); Lane 6 Nicotine (150 ng/mL) + mCRP (100 µg/mL); Lane 7 ACh (100 µM); Lane 8, ACh (10 µM) alone; Lane 9, Nicotine (150 ng/mL) alone. The mCRP induced phosphorylation of ERK1/2 (B), JNK 1/2 (C), NF-KB (D) and MAPKp38 (E). When the macrophages were pre-treated with ACh (100 and 10 µM) or Nicotine (150 ng/mL) for 8 minutes, there was a reduced ERK1/2, JNK 1/2 and NF-KB phosphorylation (n=2).

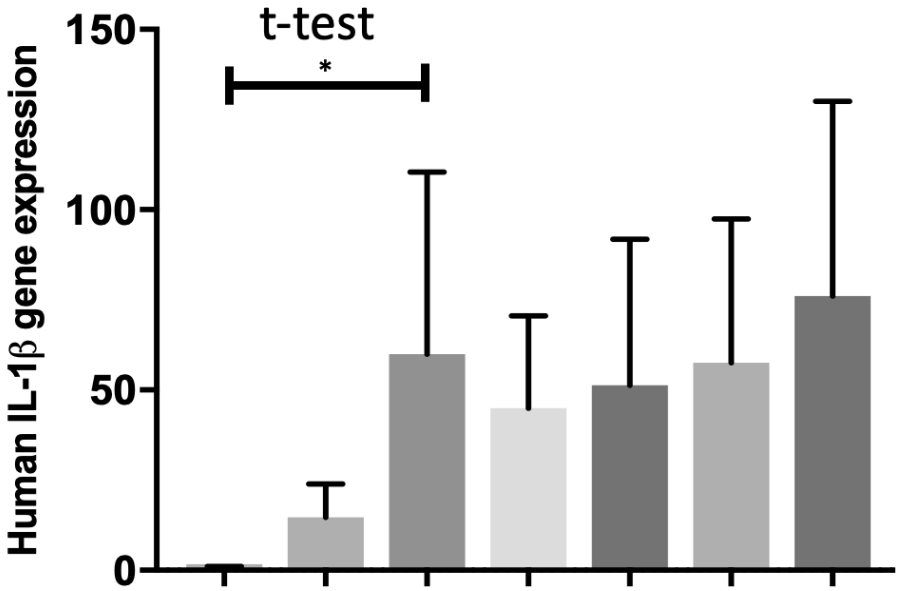
5.7 RT-PCR data demonstrated that macrophage pre-stimulation with SMI (ACh and Nicotine) was not able to reduce the mCRP induced cytokine RNA expression.

After confirming the activation of the intrinsic pro-inflammatory pathway, the next step was to determine whether the ACh was capable of reducing the release of pro-inflammatory cytokines, downregulating both the IL-1β and IL-6 gene expression. The gene expression of pro (IL-1β and IL-6) and anti (IL-10) inflammatory cytokines was determined by RNA isolated from the samples and analysed by RT-PCR. In this experiment, the TNF-α gene expression was replaced by the IL-1β gene expression, due to the short TNF-α mRNA life-span (even when there was a high TNF-α cytokine secretion), as reported in a previous study (Mijatovic et al., 2000). LPS, mCRP and SMI concentrations and time-point were the same of experiment 5.5.

As expected, there was a statistically significant upregulation of IL-1β in the mCRP-stimulated group, after 24 hours (**Fig 5.12**). Furthermore, the average IL-1β gene expression was higher in the experimental group (mCRP alone) than in the untreated macrophage and positive control group (LPS 10 ng/mL). None of the inhibitors under investigation were able to reduce this increase.

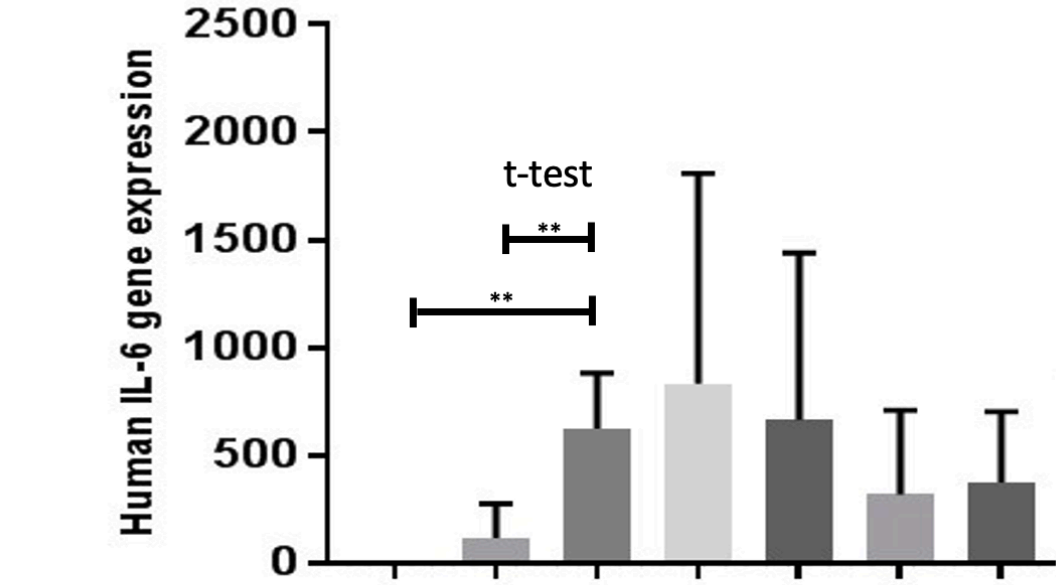
There was a statistically significant upregulation of IL-6 in the mCRP-stimulated group after 24 hours. The average IL-6 gene expression was higher in the experimental group (mCRP alone) than in the untreated macrophage group and the positive control group. No statistically significant differences were observed between the mCRP group and the group that had previously been treated with SMIs. **Fig 5.13** reports the results.

The ELISA assay data (chapter 4, paragraph 4.6) evidenced that there was no statistically significant upregulation of IL-10 in the mCRP-stimulated group after 24 hours. The data on the IL-10 gene are in line with the previous ELISA assay, which confirm that there were no differences in the IL-10 cytokine release between the untreated macrophage group and the groups stimulated with mCRP (P-value=0.3301). **Fig 5.14** reports the results.



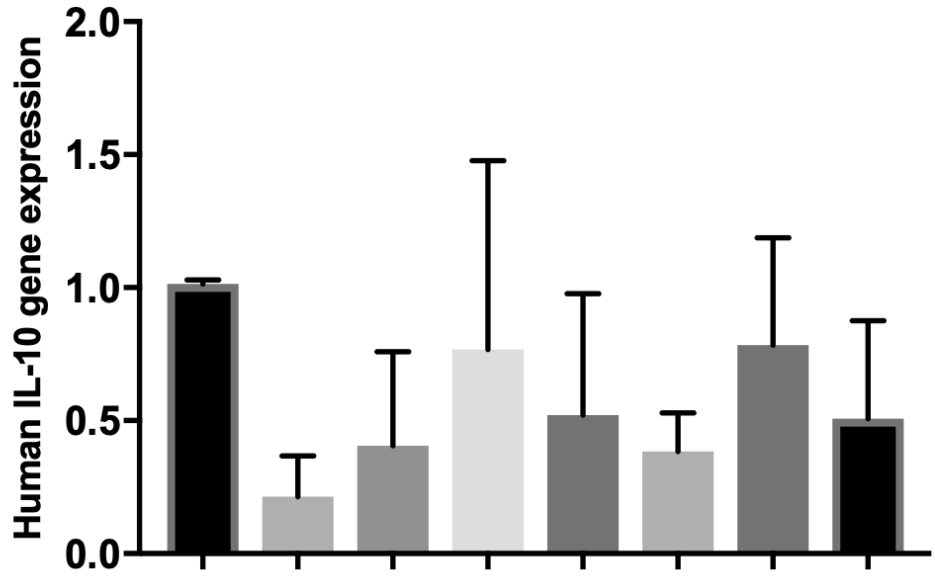
Macrophages	+	+	+	+	+	+	+
LPS 10 ng/mL	-	+	-	-	-	-	-
mCRP 100 µg/mL	-	-	+	+	+	+	+
ACh 100 µM	-	-	-	+	-	-	-
ACh 10 µM	-	-	-	-	+	-	-
Nic 150 ng/Ml	-	-	-	-	-	+	-
Nic 75 ng/mL	-	-	-	-	-	-	+

Fig 5.12. Pre-stimulation with SMIs (ACh and Nicotine) did not reduce the mCRP-induced mRNA-IL-1β expression. Following differentiation (PMA 50 ng/mL for 72 hours), the monocyte differentiate macrophages were pre-treated with ACh (100 and 10 µM) or Nicotine (150 and 75 ng/mL) for 2 hours. This was followed by mCRP stimulation at 100 µg/mL, for 24 hours. LPS (10 ng/mL for 24 hours) was used as a positive control. The IL-1β gene expression was quantified by both the TaqMan™ Fast Advanced Master Mix and the TaqMan® Gene Expression Assays. The results are shown as the average ± SD. *P-value < 0.05. The One-tailed unpaired t-test, with a 95% CI and a α error fixed at 5%, evidenced a statically significant difference (P-value<0.0296) between the untreated macrophage group and the group stimulated with mCRP. The ANOVA One-way test did not evidence any statistically significant differences between the untreated macrophage group and the mCRP group (P-value=0.7768). The ANOVA One-way test also confirmed that there were no statistically significant differences between the mCRP group alone and all the other groups previously stimulated by ACh or Nicotine (P-value=0.9944) (n=3).



Macrophages	+	+	+	+	+	+	+
LPS 10 ng/mL	-	+	-	-	-	-	-
mCRP 100 µg/mL	-	-	+	+	+	+	+
ACh 100 µM	-	-	-	+	-	-	-
ACh 10 µM	-	-	-	-	+	-	-
Nic 150 ng/Ml	-	-	-	-	-	+	-
Nic 75 ng/mL	-	-	-	-	-	-	+

Fig 5.13. Pre-stimulation with SMI (ACh and Nicotine) did not reduce the mCRP-induced mRNA-IL-6 expression. Following differentiation (PMA 50 ng/mL for 72 hours), the monocyte differentiate macrophages were pre-treated with ACh at 100 and 10 µM or Nicotine at 150 and 75 ng/mL for 2 hours. This was followed by mCRP stimulation at 100 µg/mL, for 24 hours. LPS (10 ng/mL for 24 hours) used as a positive control. The IL-6 gene expression was quantified by both the TaqMan™ Fast Advanced Master Mix and the TaqMan® Gene Expression Assays. The results are shown as the average ± SD. **P-value<0.01 *P-value < 0.05. The One-tailed unpaired t-test, with a 95% CI and a α error fixed at 5%, evidenced a statistically significant difference (P-value<0.0014) between the untreated macrophage group and the mCRP group. A statistically significant difference (P-value=0.0077) was observed between the positive control group and the mCRP group. The ANOVA One-way test did not evidence any statistically significant differences between the untreated macrophage group and the mCRP group (P-value= 0.7768). The ANOVA one-way test also evidenced that there were no statistically significant differences between the mCRP group alone and all the other groups that had previously been treated with ACh or Nicotine (n=3).



Macrophages	+	+	+	+	+	+	+	+
LPS 10 ng/mL	-	+	-	-	-	-	-	-
mCRP 100 µg/mL	-	-	+	+	+	+	+	+
ACh 100 µM	-	-	-	+	-	-	-	-
ACh 10 µM	-	-	-	-	+	-	-	-
Nic 150 ng/Ml	-	-	-	-	-	+	-	-
Nic 75 ng/mL	-	-	-	-	-	-	+	-
3H12 1:10	-	-	-	-	-	-	-	+

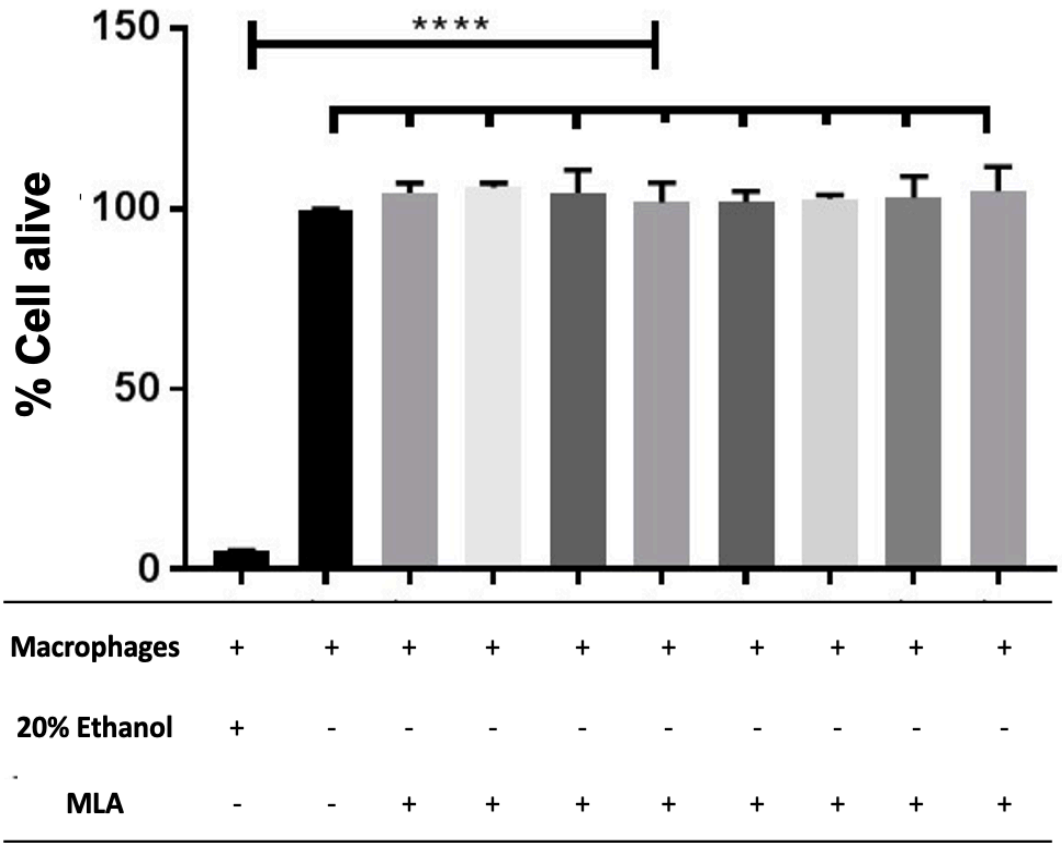
Fig 5.14. Pre-stimulation with SMI (ACh and Nicotine) did not affects the mCRP-induced mRNA-IL-10 expression. Following differentiation (PMA 50 ng/mL for 72 hours), the monocyte differentiate macrophages were pre-exposed to ACh (100 and 10 µM) or Nicotine (150 and 75 ng/mL) for 2 hours. This was followed by mCRP (100 µg/mL) stimulation for 24 hours. LPS (10 ng/mL for 24 hours) was used as a positive control. The IL-10 gene expression was quantified by both the TaqMan™ Fast Advanced Master Mix and the TaqMan® Gene Expression Assays. The results are shown as the average ± SD.*P –value < 0.05. The ANOVA One-way test, followed by Tukey's post-hoc test, with a 99.9% CI and an α error of 0.001%, did not evidence any statistically significant differences (P-value<0.0001) amongst all the groups. Furthermore, the average IL-10 gene expression was lower in the experimental groups (mCRP alone) than in the untreated macrophage group (n=3).

5.8 Methyllycaconitine (MLA) citrate toxicity analysis on U937-derived macrophages.

After observing that ACh had anti-inflammatory effects, the next step was to evaluate whether its anti-inflammatory activity was due to α7nAChR. As reported in literature, MLA is a selective α7nAChR antagonist (Sun et al., 2013a; Krafft et al., 2017; Patel et al., 2017; Zou et al., 2017). To support the aforementioned, MLA was used to block the α7nAChR activation.

MLA cytotoxic activity was evaluated first. A cell viability assay was performed to determine whether MLA had cytotoxic effects that could lead to cell death after 24-hours of stimulation. The macrophages were incubated with MLA for 24 hours, in a range between 0 and 200 μM, i.e., two-fold the highest ACh working concentration (100 μM) used in the previous experiments to determine a safe MLA concentration.

As reported in **Fig 5.15** and **Table 5.4,** MLA did not show any cytotoxic effects in a range between 0-200 μM. Therefore, on the basis of these results, a working concentration of 200 μM was selected to assess whether MLA alone or in combination with ACh and Nicotine, induced pro-inflammatory effects on U937 macrophages.



MLA Concentration	Pos contr	0 µM	1,5 µM	3,1 µM	6,2 µM	12,5 µM	25 µM	50 µM	100 µM	200 µM
% Viable cells	5.20	99.99	104.43	105.93	104.43	101.84	101.88	102.82	103.09	104.91

Fig. 5.15. and **Table 5.4. Methyllycaconitine (MLA) citrate toxicity analysis on U937-derived macrophages.** Following differentiation (PMA 50 ng/mL for 72 hours), the monocyte differentiate macrophages were pre-exposed to a media containing RPMI 1640 with L-glutamine (1640 medium Lonza), supplemented with 10% FBS (F9665 Sigma®) and MLA (from 1.5 µM to 200 µM). Ethanol (20% total volume for 24 hours) was used as a positive control. Cell viability was evaluated by the CellTiter-Blue®Cell Viability Assay (Promega). The results are shown as the average ± SD. ****P-value< 0.0001. The results herein reported confirm that MLA did not have cytotoxic effects in a range between 0-200 µM. The ANOVA One-way test, along with Tukey's post-hoc test, confirmed that there was a statically significant difference (P-value<0.0001) between the positive control group (20% Ethanol) and the untreated macrophage group. There was no statistically significant difference between the untreated macrophage group and all groups stimulated by MLA, at a concentration ranging from 0 µM to 200 µM (P-value =0.8943). (n=3).

5.9 ACh and Nicotine with and without MLA as a method to evaluate pro-inflammatory effects on U937 macrophages.

After having determined that MLA had no cytotoxic effect, MLA alone or together with SMIs (ACh and Nicotine), were tested to corroborate that they did not induce any pro-inflammatory activity on U937 macrophages. The MLA working concentration used for these experiments, i.e., 200 and 100 μM were based on the previous experiment reported in chapter 5, paragraph 5.8. These two MLA concentrations were identical or two-fold the highest ACh working concentration of 100 μM used in the previous experiment. The ACh and Nicotine concentration and time-points were the same as those in experiment 5.4. The TNF-α pro-inflammatory cytokine level was evaluated by ELISA assay. The TNF-α pro-inflammatory cytokine was selected on the basis of the same concepts expressed in chapter 4, paragraph 4.6.

As reported in **Fig 5.16**, the TNF-α cytokine concentrations between the untreated macrophage, positive control and the MLA stimulated groups at 200 μM and 100 μM, with or without ACh (100 μM and 10 μM) or Nicotine (150 ng/mL and 75 ng/mL) after 24 hours, were evaluated. No statistically significant difference was observed between the untreated macrophage group and the groups stimulated by MLA alone or in combination with both ACh or Nicotine. There was a statistically significant difference between the untreated macrophage group and the positive control, as well as the positive control and the SMI groups.

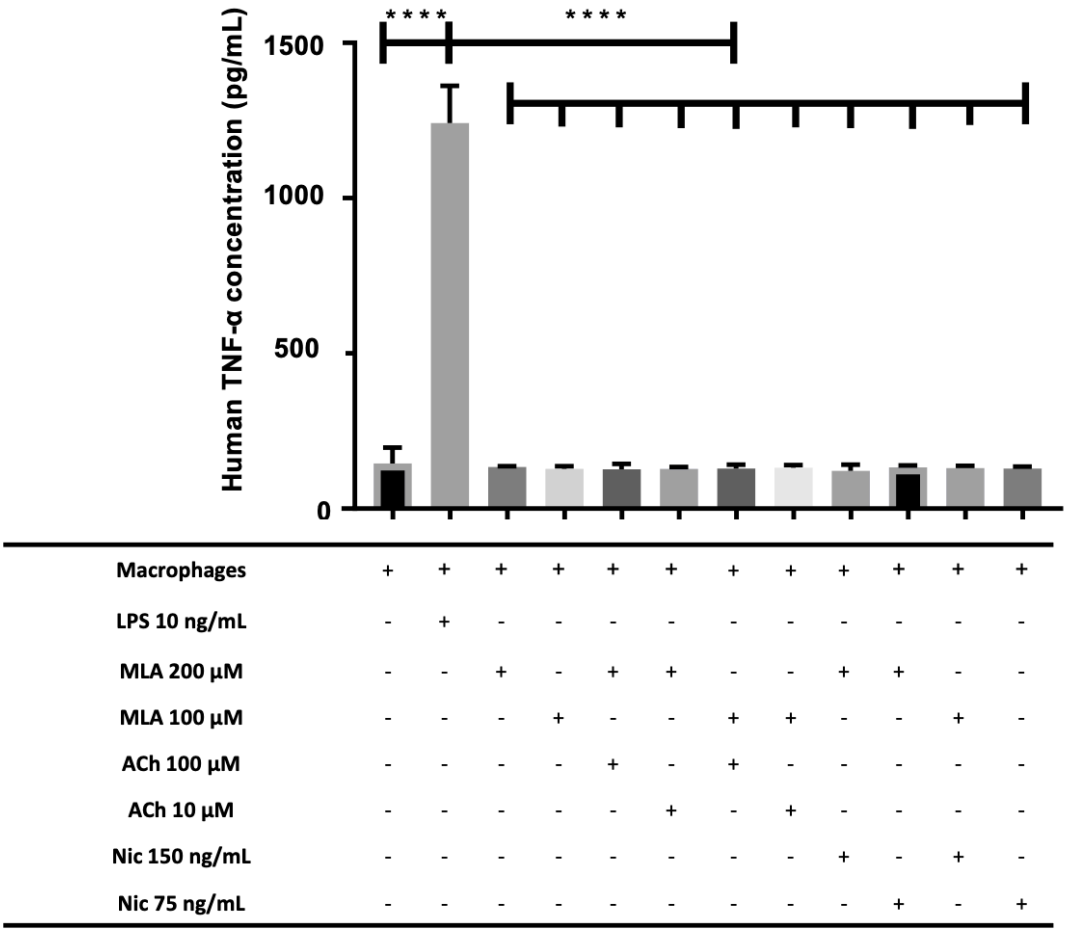


Fig 5.16. MLA alone or together with SMIs did not induce any pro-inflammatory activity on U937 macrophages. The macrophages were pre-exposed to a media containing RPMI 1640 with L-glutamine (1640 medium Lonza) supplemented with 10% of FBS (F9665 Sigma®) and MLA (200 and 100 µM), for 2 hours. This was followed by ACh stimulation at 100 and 10 µM, or Nicotine, at 150 and 75 ng/mL, for 24 hours. LPS alone (10 ng/mL) was used as a positive control for 24 hours. The TNF-α production was quantified by ELISA assay (R&D System). The results are shown as the average ± SD. ****P-value< 0.0001. The One-way ANOVA test, followed by Tukey's post-doc test, with a 99.9% CI α error fixed at 0.001%, evidenced no statistically significant differences between the untreated macrophage group and groups stimulated by MLA alone or in combination with both ACh or Nicotine. The only statistically significant difference (P-value<0.0001) was observed between the positive control group and all the other groups. (n=3).

5.10 MLA did not have any general LPS anti-inflammatory effect on macrophages.

After having demonstrated that MLA at 200 and 100 μM did not have any pro-inflammatory activity on macrophages and before testing MLA specific activity to block α7nAChR activation, it became essential to assess whether MLA was characterised by anti-inflammatory proprieties. In this experiment the U937 differentiated macrophages were treated with LPS at 10 ng/mL, both with or without 200 μM of MLA, for 24 hours. The MLA concentration was selected on the basis of the data obtained in experiment 5.9. An MLA pre-treatment time point of 2 hours was the best time-point obtained in experiment 5.4. The TNF-α pro-inflammatory cytokines used were selected on the basis of the same notions expressed in chapter 4, paragraph 4.6. The TNF-α production in macrophages was assessed after LPS stimulation at a concentration of 10 ng/mL, for 24 hours.

The ELISA assay demonstrated that MLA alone did not reduce the TNF-α in pro-inflammatory cytokine release. As shown in **Fig 5.17,** there was no statistically significant difference amongst the LPS group and the groups with or without pre-MLA treatment. The MLA activity effects were neither pro-inflammatory nor anti-inflammatory.

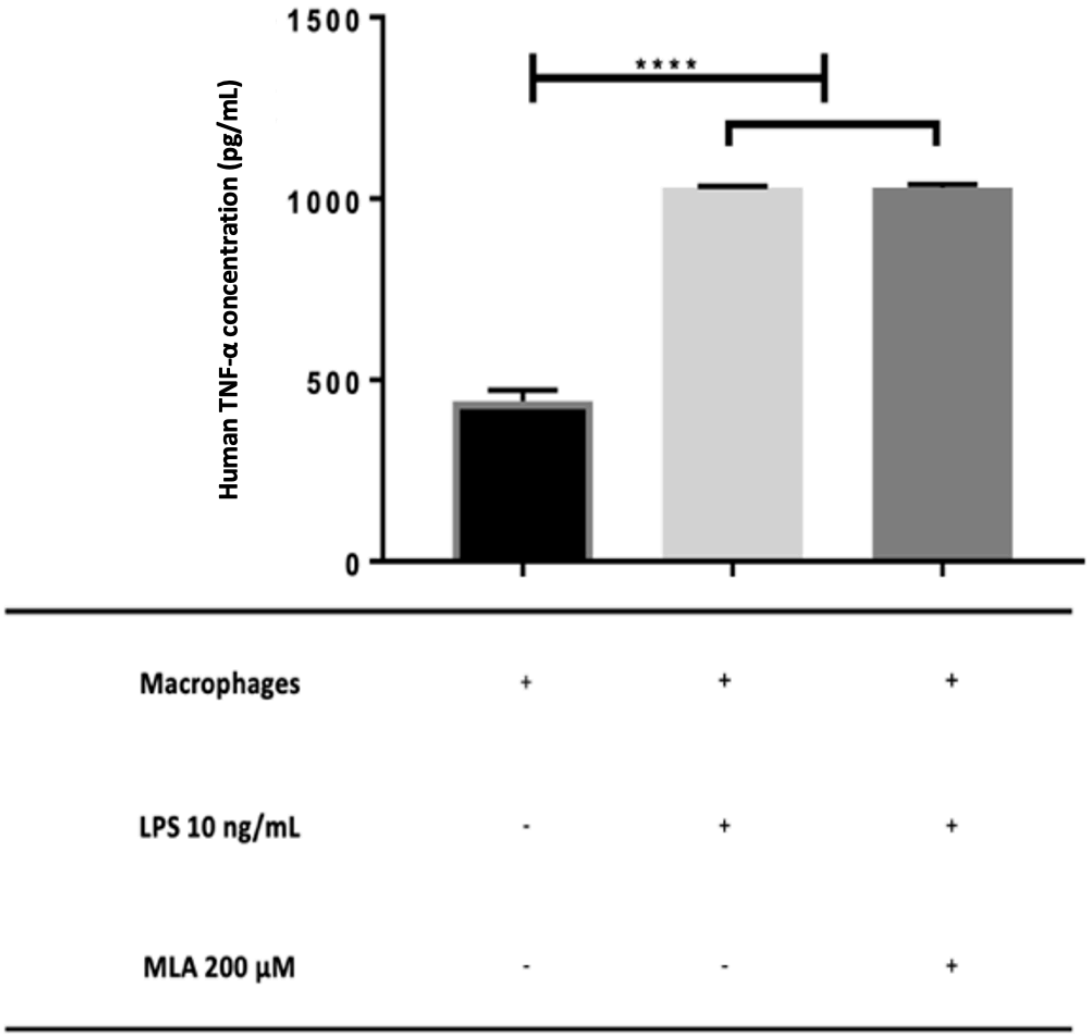


Fig 5.17. MLA did not reduce lipopolysaccharide (LPS) inflammatory effect in macrophages. The monocyte differentiated macrophages were pre-exposed to a media containing RPMI 1640 with L-glutamine (1640 medium Lonza) supplemented with 10% of FBS (F9665 Sigma®) and MLA (200 µM) for 2 hours, then stimulated by LPS (10 ng/mL), for 24 hours. LPS alone, at 10 ng/mL, was used for 24 hours as a positive control. The pro-inflammatory cytokine TNF-α was estimated by ELISA assay (R&D System). The results are shown as the average ± SD. ****P-value<0.0001. The One-way ANOVA test evidenced a statically significant difference (P-value<0.0001) amongst the untreated macrophage group and the LPS stimulated groups (442.2 vs 1030 pg/mL). No statistically significant difference was observed amongst the LPS stimulated groups, with and without pre-MLA treatment (P-value= 0.9989). (n=3).

5.11 Studying the MLA association according to the pro-inflammatory response.

The effect MLA had on inflammation was also tested by the western blot assay. To this aim, the MLA-response activation of MAPKp38 and NF-KB was evaluated. This was based on the data reported in paragraph 5.6, which confirmed that when macrophages were stimulated by ACh and Nicotine only, there was an increase in MAPKp38 and/or NF-KB phosphorylation.

As shown in **Fig 5.18**, LPS stimulation increased the phosphorylation of both MAPKp38 and NF-KB. Whereas in all the MLA stimulated groups, with or without SMIs (ACh or Nicotine), phosphorylation was minimal or completely absent.

The trend herein reported evidenced that MLA alone or in combination with ACh or Nicotine was not able to activate any signalling pathways that may have been associated with a pro-inflammatory response.

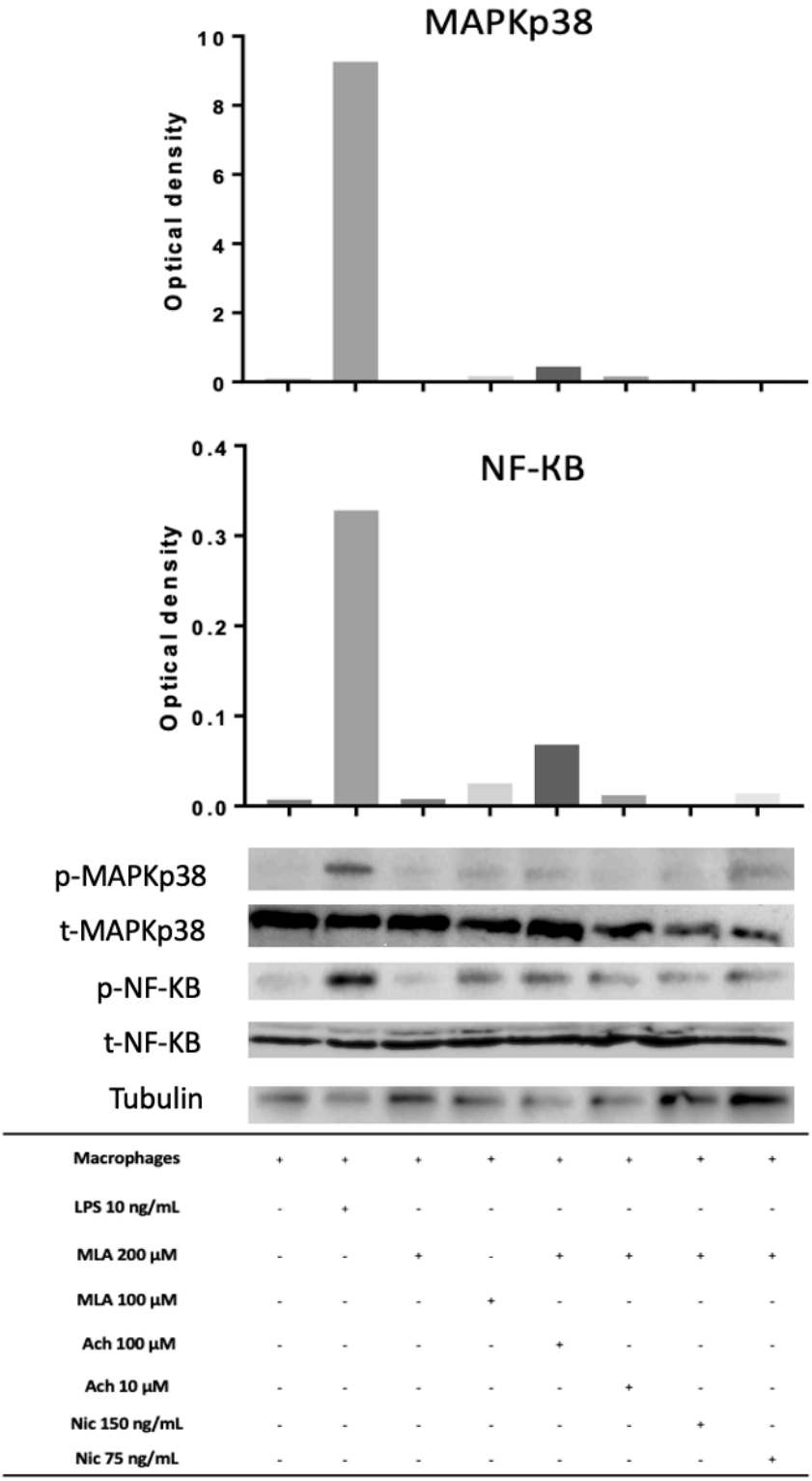


Fig 5.18. MLA alone or in combination with ACh or Nicotine did not activate any signalling pathways. Macrophages were pre-stimulated with a media containing RPMI 1640 with L-glutamine (1640 medium Lonza) supplemented with 10% of FBS (F9665 Sigma®) with or without MLA (200 µM), ACh (100 and 10 µM) or Nicotine (150 and 75 ng/mL), for 8 minutes. LPS alone at 10 ng/mL for 8 minutes, was used as a positive control. Lane 1, untreated macrophage; Lane 2, LPS (10 ng/mL); Lane 3 and 4, MLA (200 and 100 µM) Lane 5 (MLA 200 + ACh100 µM), Lane 6 (MLA 200 + ACh10 µM), Lane 7 (MLA 200 + Nic 150 ng/mL), Lane 8 (MLA 200 + Nic 75 ng/mL). As reported in this figure, phosphorylation was minimal or completely absent in all groups treated with MLA (with or without SMIs ACh or Nicotine). (n=1).

5.12 MLA did not reverse ACh anti-inflammatory capacity but did reduce TNF-α release.

Therefore, so as to determine if the anti-inflammatory effects of ACh were mediated by α7nAChR, the next step involved assessing whether MLA could reverse the ACh anti-inflammatory activity. MLA and the SMIs (ACh or Nicotine) were incubated together to analyse whether MLA was able to block or reduce the anti-inflammatory activity of a selected ACh SMI.

The macrophages were stimulated by MLA (200 μM) for 2 hours and then ACh (100 and 10 μM) or Nicotine (150 and 75 ng/mL) and mCRP 100 μg/mL. Both SMIs and mCRP at the same time-point and concentration as those used in experiment 5.5. The TNF-α cytokine concentration was quantified by ELISA assay. The TNF-α pro-inflammatory cytokines were chosen on the basis of the same notions expressed in chapter 4, paragraph 4.6.

As reported in **Fig 5.19,** mCRP increased the TNF-α release substantially. There was a substantial TNF-α inhibition in the group pre-stimulated by ACh at 100 μM. Surprisingly, the results of this experiment evidenced that not only did MLA and ACh used together (100 and 10 μM) not reverse ACh anti-inflammatory capacity, but rather emphasised it. **Fig 5.19**. reports the results.

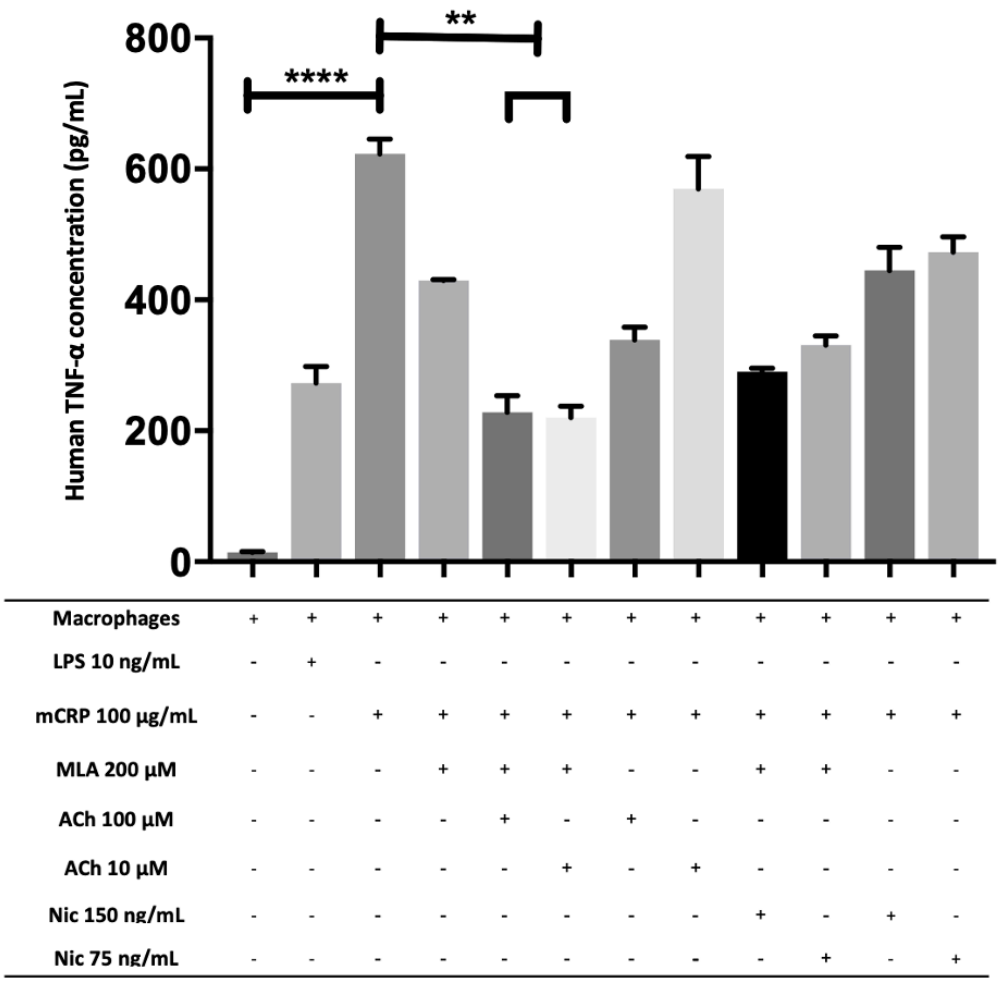


Fig 5.19. MLA did not reverse ACh anti-inflammatory capacity however it did decrease TNF-α release. Macrophages were pre-stimulated with a media containing RPMI 1640 with L-glutamine (1640 medium Lonza) supplemented with 10% of FBS (F9665 Sigma®) and MLA (200 µM) for 2 hours followed by another 2 hours of SMI stimulation (ACh or Nicotine). Then, macrophages were stimulated by mCRP (100 µg/mL), for 24 hours. LPS at a concentration of 10 ng/mL for 24 hours was used as a positive control. The TNF-α production was quantified by ELISA assay (R&D System). The results are shown as the average ± SD. ****P-value < 0.0001, **P-value < 0.01. As reported in the figure, surprisingly MLA did not reduce ACh anti-inflammatory activity but rather led to an increase. The One-way ANOVA test, followed by Tukey's post-doc test confirmed a statistically significant reduction (P-value=0.0078) in TNF-α release when MLA (200 µM) and ACh (100 and 10 µM) were combined. (n=3).

5.13 Discussion.

It has been reported that monomeric-CRP (mCRP) is generated by activated platelets near the atherosclerotic plaques when nCRP bind to membrane lipids and come into contact with derivates of phosphatidylcholine (PC) exposed on the cell membrane (Eisenhardt et al., 2009b). This mCRP has been observed close to inflammation tissue (Eisenhardt et al., 2009b; Slevin et al., 2010), along with an increase in $CD68^{+}$ monocytes/macrophages (Thiele et al., 2014a), associated to a strong pro-inflammatory activity and pro-inflammatory cytokine release (Slevin et al., 2015; McFadyen et al., 2018). An increase in pro-inflammatory cytokines is frequently associated to an increase in non-neuronal serum Acetylcholine (ACh) (Zhang et al., 2016). CAIP release ACh to attenuate the pro-inflammatory mediator release (Duris et al., 2017) and counterbalance the potential negative effects of the pro-inflammatory cytokine release during the acute-phase response (Gruys et al., 2005; Jain et al., 2011). Therefore, this project aimed at evaluating the mCRP immune regulatory properties. This was done by using an *in vitro* inflammation model with monocyte-like U937 cells differentiated into macrophages. Furthermore, taking into consideration that both ACh and Nicotine showed anti-inflammatory effects (Lakhan and Kirchgessner, 2011; Han et al., 2017), a series of experiments were carried out during this project to establish whether and how ACh or Nicotine could reduce mCRP pro-inflammatory activity.

The results reported during this PhD project showed that, *(I)* ACh and Nicotine were not cytotoxic to the U937-derived macrophages, *(II)* did not increase cytokine release, and *(III)* did not activate any signalling pathways that could have been associated with a pro-inflammatory response. However, the results of the experiments carried out in this project are in disagreement with data from studies which report that both ACh and Nicotine have strong anti-inflammatory proprieties in LPS stimulated macrophage cells (Yoshikawa et al., 2006b; Liu et al., 2015a), as reported in **Fig. 5.6** and **Fig. 5.7**. This discrepancy might be explained by the fact that the LPS and SMI concentrations and time-point stimulation in these two studies differed substantially (Yoshikawa et al., 2006b; Liu et al., 2015a).

Literature data report that once MAPKp38, JNK1/2, ERK1/2 and NF-KB are activated they increase pro-inflammatory mediator release (Kyriakis and Avruch, 2001; de Oliveira et al., 2017; Lim et al., 2018). Some findings also indicate that when ECs are stimulated by mCRP (Li et al., 2014), these cells increase the secretion of some pro-inflammatory mediators, such as IL-6, ICAM-1 and VCAM-1, through the activation of MAPKs and NF-KB intrinsic pathways (Yang et al., 2008; Eisenhardt et al., 2009b; Yong and Cao, 2020). However, a previous study did not identify any intrinsic pathways triggered by mCRP on macrophages (Eisenhardt et al., 2011). This led to the assessment of whether these signalling pathways could also be triggered in U937 monocyte-differentiated macrophages after mCRP treatment, in this project.

The results herein reported are in agreement with the statement that mCRP increases pro-inflammatory levels of TNF-α and IL-6 cytokines (**Fig 5.9**) through ERK 1/2, JNK 1/2, MAPKp38 and NF-ĸB phosphorylation (**Fig 5.11**). ACh alone, at 100 and 10 μM, increase MAPKp38 and NF-ĸB phosphorylation. Nicotine alone at 150 ng/mL, increases only the MAPKp38 phosphorylation. However, none of the aforementioned SMIs were associated with an increase in JNK1/2 and ERK1/2 phosphorylation or pro-inflammatory cytokine release (**Fig 5.11**). Furthermore, although pre-treatment with either ACh or Nicotine was able to reduce ERK1/2, JNK1/2 and NF-KB phosphorylation caused by mCRP, ACh was the only SMI capable of limiting the increase of mCRP induced pro-inflammatory cytokines (**Fig 5.11**).

The RT-PCR experiments herein reported showed that mCRP increases the IL-1β and IL-6 gene expression. However, none of the SMI inhibitors under study were able to reduce significantly either the IL-1β or IL-6 RNA expression in this model of inflammation (**Fig 5.12** and **Fig 5.13**). All results presented during this project validated that SMIs were able to reduce IL-1β gene expression, even if statistical significance was not reached. This was also observed when ACh was used at a concentration of 100 μM (24.79 % the highest reduction). Of particular interest is that fact that although ACh is able to reduce the pro-inflammatory protein concentration, it does not downregulate IL-1β and IL-6 gene expression. A possible explanation for this can be found

in the literature. Indeed, Clark et al. (2009) reported that when inflammatory stimuli, such as IL-1, TNF, or LPS, activate the transforming growth factor β–activated kinase 1 (TAK1) this macro complex protein triggers the MAPKp38 signalling pathway. Once MAPKp38 is activated, it is able to increase the MK2/3 and the mRNA decay-promoting protein tristetraprolin (TTP) phosphorylation (Hitti et al., 2006). Moreover, it has been reported that TTP phosphorylation leads to the loss of binding to AU-rich elements (AREs), increasing cytokine mRNA stability and translation (Khabar, 2014). Borovikova et al. (2000) reported that ACh reduced pro-inflammatory cytokine release through a post-transcriptional event, whilst Waldburger et al. (2008) stated that ACh reduced the half-life of IL-6 mRNA from 13.8 hours to 6.5 hours. Therefore, taking in consideration that both ACh and Nicotine were able to reduce the ERK and JNK intrinsic pathway (**Fig 5.11**), without reducing the MAPKp38 phosphorylation or the interleukin gene expression (**Fig 5.11**), the reduction of the protein cytokine concentration might well depend on the capacity ACh has to intervene at a post-transcriptional level (**Fig 5.20**), through the involvement of microRNA-132 (miR-132), in line with what was previously reported by Liu et al. (2015c).

Although ACh and Nicotine have been reported to have anti-inflammatory proprieties (Lakhan and Kirchgessner, 2011; Mihara et al., 2017) the results of the experiments carried out in this project showed that only ACh was able to reduce mCRP pro-inflammatory activity (**Fig 5.9**). Nicotine did not play an anti-inflammatory role. This is most likely connected to the fact that Nicotine itself can increase nCRP expression through the nAChR and nAChR-MAPK signalling pathways on U937-monocyte differentiated macrophages (Mao et al., 2012). This increase may also interfere with the potential anti- inflammatory activity of Nicotine against the mCRP (**Fig 5.21**).

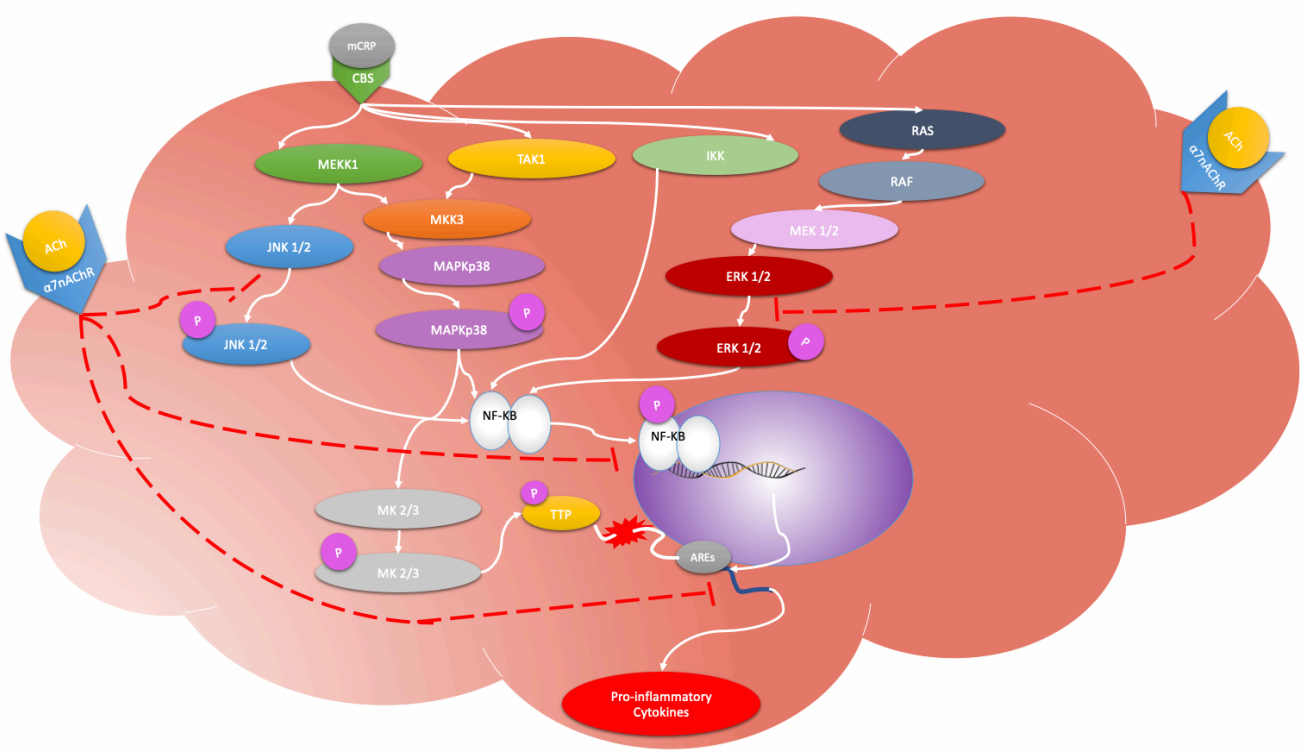


Fig 5.20. ACh reduced the pro-inflammatory cytokines concentration due to its ability to reduce the phosphorylation of the ERK/JNK/ NF-KB intrinsic pathway. Monomeric-CRP promote the activation of the (TAK1) signalling complex. TAK1 then activates MKK3 and IKK. MKK3 then lead to MAPKp38 phosphorylation, which, in turn induce MK2/3 phosphorylation and the nuclear translocation of the transcription factor NF-KB. mCRP can also promote MEKK1 activation. MEKK1 then activates JNK 1/2. Then JNK 1/2 phosphorylated cause the nuclear translocation of the NF-KB transcription factor. mCRP can also trigger the Ras-Raf-MEK-ERK1/2 pathway. When ERK1/2 are activated this activation facilitates the nuclear translocation of the NF-KB transcription factor. NF-KB increase the mRNA expression of several pro-inflammatory cytokines, which can be translate into protein when MK2/3 (previously phosphorylated by MAPKp38) and TTP also become phosphorylated. The use of ACh to block JNK1/2 and ERK1/2 phosphorylation can also reduce the nuclear translocation of the NF-KB transcription factor. ACh is not able to block MAPKp38 phosphorylation, which promotes NF-KB nuclear translocation. However, although NF-KB increase the pro-inflammatory gene expression (mRNA), ACh can intervene at a post-transcriptional level, reducing the life of mRNA.

After observing ACh anti-inflammatory effects, the next step was to evaluate whether ACh anti-inflammatory activity could be triggered through the activation of α7nAChR. Over the past few years, numerous studies have postulated that the activation of α7nAChR and/or the use of the α7nAChR agonist (ACh and Nicotine) could be a useful and innovative treatment for several medical conditions (Liu et al., 2015b; Lombardo and Maskos, 2015; Han et al., 2017; Xu et al., 2019). For example in stroke, where the presence of mCRP has been confirmed (Slevin et al., 2010), an *in vivo* study also reported that α7nAchR activation could reduce sensorimotor deficits, lesion size, neural decease, the presence of $CD68^{+}$ cells, the ratio between M1 and M2 microglia and the NF-KB activity (Han et al., 2014).

So as to evaluate α7nAchR activation, methyllycaconitine (MLA), recognised as a selective α7nAchR antagonist (Carroll et al., 2007; Patel et al., 2017; Krafft et al., 2017; Zou et al., 2017), was tested to observe whether it could reduce ACh anti-inflammatory properties. The results confirmed that *(I)* in appropriate concentrations (from 0 to 200 μM) MLA was not cytotoxic to the U937 derived macrophages *(II)* alone or in combination with ACh and Nicotine MLA did not induce any pro or anti-inflammatory effects on U937 macrophages. However, contrary to expectations, MLA did not reverse the ACh anti-inflammatory capacity but enhanced the ACh anti-inflammatory proprieties, reducing TNF-α release even further **Fig 5.19**. These results suggest that MLA could also potentially play an anti-inflammatory role, as previously reported for microglia cells (Thomsen and Mikkelsen, 2012). Therefore, as the data from the experiments showed that both ACh and MLA had the same anti-inflammatory activity on macrophages, the idea that α7nAchR is in some way involved in the ACh anti-inflammatory activity cannot be rejected as both ACh and MLA bind to α7nAchR. Moreover, it may be that the anti-inflammatory activity of ACh is mediated by another receptor, i.e., the muscarinic Acetylcholine receptor (mAChR) (Fujii et al., 2017).

Muscarinic receptors are extensively diffused in human body tissue and facilitate various physiological tasks, depending to their location and

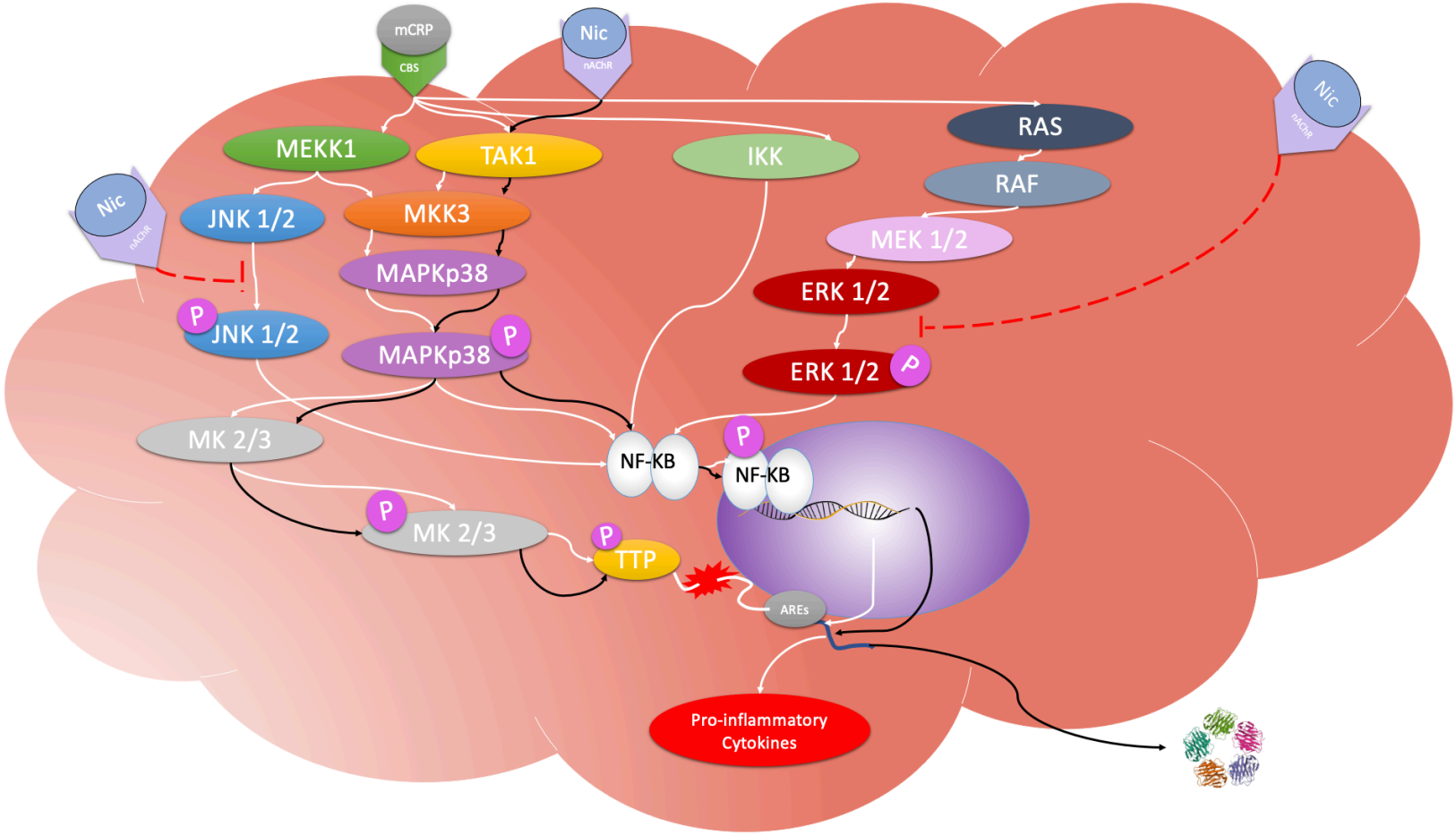


Fig 5.21. Nicotine did not reduce the pro-inflammatory cytokines concentration due to its ability to activate nAChR-MAPK signalling pathways. As herein reported, it is most likely that Nicotine does not reduce pro-inflammatory cytokine release, as by the activation of the TAK1-MEKK3-MAPKp38-NF-KB signalling pathway it is able to synthesis and release nCRP.

receptor subtype (Caulfield and Birdsall, 1998). Literature reports that there are 5 different mAChR subtypes (from M0 to M5) (Bonner, 1989), which allow the neurotransmitter ACh to bind and carry out their physiological functions (Kellar et al., 1985) and that the mAChR subtypes are also associated with a strong anti-inflammatory and antioxidant activity (Frinchi et al., 2019). All five mAChRs have been confirmed in immune system cells, such as B cells, macrophages and DCs (Kawashima et al., 2007). Indeed, Koarai et al. (2012) reported that human monocytes express all 5 mAChR subtypes but that the expression of M1, M2 and M3 receptors on monocyte-differentiated macrophages are considerably higher than on monocytes. Therefore, the current bibliography could support the hypothesis that the anti-inflammatory effect of ACh may not only be mediated by the α7nAchR, but also by at least one of the aforementioned mAChR.

5.14 Key findings:

The key findings of the experiments described in this chapter are:

- Before testing the anti-inflammatory activity of both ACh and Nicotine, these two SMIs were tested by a cell viability and cell apoptosis assay, to assess whether SMIs had cytotoxic effects that might lead to cell death. It was observed that neither ACh nor Nicotine triggered pro-inflammatory activity on monocyte U937-differentiated macrophages.

- The second step was that of carrying out experiments to assess whether mCRP pro-inflammatory activity could be blocked, or at least reduced, by one of these two SMIs or by the use of the anti-mCRP antibody. The result herein reported confirmed that the mCRP pro-inflammatory activity were limited when the macrophages were previously pre-treated with ACh, at concentration of 100 μM.

- The activation of MAPKp38, JNK, ERK1/2 and NF-KB are strong players involved in the increase of pro-inflammatory cytokines on macrophages (Liu et al., 2003; Moens et al., 2013; de Oliveira et al.,

2017; Lim et al., 2018). The results herein reported showed that mCRP is able to increase the phosphorylation of ERK1/2, JNK, MAPKp38 and NF-KB. Furthermore, although both ACh and Nicotine reduced ERK 1/2, JNK 1/2, NF-KB phosphorylation, only ACh was able to reduce the pro-inflammatory cytokine release through a pre- and post-transcriptional event.

- Another objective of this study was to assess whether ACh had anti-inflammatory activity on macrophages through the activation of the α7nAchR. To this aim α7nAchR antagonist MLA was used. Contrary to expectations, this α7nAchR antagonist did not reduce the ACh anti-inflammatory activity but rather exacerbated it.

5.15 Summary.

During the first phase of the inflammation process the increase in pro-inflammatory cytokines has been associated with an increase in non-neuronal serum ACh, due to the activation of CAIP. Several studies have confirmed that this neurotransmitter has anti-inflammatory properties, during the last few years. This prompted the study of whether, two cholinergic compounds, i.e., ACh and Nicotine, were able to reduce the mCRP pro-inflammatory effects.

The aforementioned experiments showed that mCRP has pro-inflammatory activity and that its capacity to increase pro-inflammatory cytokine release is associated with an increase in the phosphorylation of both the MAPK and NF-KB signalling pathways. Contrary to Nicotine, the SMI ACh exerted anti-inflammatory activity against mCRP and these anti-inflammatory proprieties have been associated with the capacity of ACh to intervene at both a pre- and post-transcriptional level.

In conclusion, specific molecules, such as ACh or other SMIs of similar structure, potentially have a role as inhibitors for mCRP pro-inflammatory activity.

CHAPTER 6: GENERAL DISCUSSION, CLOSING REMARKS, FUTURE WORK AND LIMITATIONS.

CHAPTER 6: GENERAL DISCUSSION.

The research carried out during this project focused on obtaining a better understanding of the pro-inflammatory role of mCRP, as well as its downstream inflammasome signalling pathways and potential treatments. A series of experiments were carried out to establish if and how mCRP is able to promote inflammation activity. The experiments described ranged from dot blot analysis to *in vitro* studies and analyses as to potential signalling on monocyte-derived macrophages, through analyses of the genetic effects of mCRP. This project provided novel data on the effects of mCRP isoform in an inflammation model, based on monocyte-derived macrophages and the potential contribution of the small molecule inhibitor Acetylcholine (ACh) as a possible inhibitor of inflammatory mCRP.

6.1 How ACh affects the dissociation of nCRP to mCRP.

Inflammation is the first stage of the wound healing process (Chen et al., 2018) which induces an increase of several pro-inflammatory mediators (Freire and Dyke, 2013; Čejková et al., 2016) and APPs, like nCRP (Gans et al., 2015; Zhou et al., 2016). nCRP is a protein, made up of five different monomers (Osmand et al., 1977), assembled through disulfide bonds (Goodman et al., 1996) and symmetrically positioned around a central hole (Thompson et al., 1999; Pepys and Hirschfield, 2003; Du Clos and Mold, 2004). Under physiological conditions, nCRP maintains its pentameric form (Eisenhardt et al., 2009a). However, recent literature has reported that the stability of nCRP could be alternated in some *in vitro* and *in vivo* conditions (Salazar et al., 2014). This prompted the study of two different dissociation methods for nCRP in mCRP.

The results reported in chapter 3 evidence that the stability of nCRP is compromised when it is immersed in a buffer (**Fig. 3.2**) with a high urea concentration (8 M) in the presence of EDTA (10 mM) (Potempa et al., 1983; Potempa et al., 2015). Moreover, as previously reported by Taylor and Van Den Berg (2007), the results confirmed that the dissociation from nCRP to

mCRP could also be acquired by warming the nCRP to a temperature of +70 °C for one hour (**Fig. 3.3**). However, it was observed that warming the nCRP had a strong influence on the capacity the anti-mCRP3H12 antibody had to bind to the mCRP pro-inflammatory form (**Fig. 3.3**).

The last decade has witnessed the publication of several studies reporting that, when nCRP is dissociated into mCRP, the monomer obtained has strong pro-inflammatory proprieties (Tarek et al., 2006; Molins et al., 2008; Potempa et al., 2015), potentially associated with chronic kidney disease (Schwedler et al., 2003), thrombosis in atherosclerosis (Eisenhardt et al., 2009b) and neurological degradation and/or dementia (Slevin et al., 2015) and macular degeneration (Chirco et al., 2016). The well-defined connection between nCRP-mCRP dissociation and inflammation implies that inhibition of this separation is a realistic pharmacological approach for a wide spectrum of disorders correlated to mCRP and inflammation (Thiele et al., 2015). Previously published results state that 1,6-bis(phosphocholine)-hexane (1,6-bis-PC) is the only nCRP-mCRP dissociator inhibitor (Thiele et al., 2014a). Experiments were carried out to determine whether the use of the neurotransmitter ACh, which is similar in structure to 1,6-bis-PC (Slevin et al., 2018), is able to prevent this dissociation. This hypothesis was further supported by a previous study which confirmed that nCRP induced lower ACh levels in plasma via a capture process (Nazarov et al., 2007).

The results herein confirmed that ACh was not able to block nCRP-mCRP dissocation (**Fig. 3.4** and **Fig. 3.5**). This most likely occurred as the SMIs might bind to nCRP at a site where dissociation does not take place. Furthermore, in the computational docking analysis, it appears that the SMIs tested were not able to inhibit the nCRP dissociation in subunits. This might be due to a lower binding activity or because the SMIs are sequestered in a different binding pocket as shown in **Fig. 3.4.** Furthermore, it seems that the energy bond of ACh compared to 1,6-bisPC, is insufficient for a stable interaction, despite its similarity with 1,6-bisPC. This limitation and the difficulty of finding other SMIs that might be able to avoid the nCRP-mCRP dissociation, as reported by

Pepys et al. (2006) and Caprio et al. (2018) prompted the assessment of the potential anti-inflammatory activity of both ACh and Nicotine, that Borovikova et al. (2000) and Rosas-Ballina and Tracey (2009) confirmed to exert a strong anti-inflammatory activity on monocyte-derived macrophages.

6.2 Assessment of whether mCRP stimulation induces monocyte-derivate macrophage pro-inflammatory activity.

Over the years several studies have confirmed the finding of local deposits of mCRP, monocytes and macrophages in ischemic stroke (Slevin et al., 2010; Chiba and Umegaki, 2013), chronic kidney disease (Schwedler et al., 2003; Lee et al., 2013; Guiteras et al., 2016) Alzheimer (Feng et al., 2011; Slevin et al., 2017; Mammana et al., 2018) and infarcted myocardial tissue (Nahrendorf et al., 2010; Thiele et al., 2014b; O'Rourke et al., 2019). This promoted a series of experiments to determine whether mCRP could be involved in the physiopathology of the aforementioned pathologies, by increasing the U937 monocyte and U937monocyte-derived macrophage pro-inflammatory cytokine production.

The data reported in chapter 4 suggested that monocytes are immune system cells that duplicate very quickly (**Fig. 4.1**). However, these cells do not release pro-inflammatory TNF-α cytokines when stimulated by LPS (**Fig. 4.2**). Furthermore, even if some authors have shown that mCRP is able to bind with both αvβ3 and α4β1 integrins expressed on the U937 monocyte cell line (Fujita et al., 2014), the results obtained in this study indicate that this binding did not increase the pro-inflammatory TNF-α cytokine concentration (**Fig. 4.2**). This most likely takes place because, as previously reported, monocytes are unable to release pro-inflammatory cytokines (Wang et al., 1997; Hur et al., 2004). Therefore, it was decided to differentiate the monocytes into U937 monocyte derived macrophages to produce a valid *in vitro* inflammation model.

The results herein reported showed that U937 monocytes can differentiate into macrophages when treated with PMA at a concentration of 50 ng/mL for 72

hours (**Fig. 4.3, Fig. 4.4** and **Fig. 4.5**). After differentiation, macrophages increase pro-inflammatory cytokine secretion (**Fig. 4.6** and **Fig. 4.7**). Moreover, these cells were able to up-regulate IL-1β gene expression when they were exposed to mCRP, at a concentration of 100 μg/mL, for 12 and 24 hours (**Fig. 4.8**). The increase in IL-1β gene expression was found to be directly related to an increase in pro-inflammatory cytokines, i.e., TNF-α and IL-6 (**Fig. 4.9**) but not IFN-γ (**Fig. 4.10**). Indeed, IFN-γ is not released even after LPS stimulation (**Fig. 4.10**) probably due to the fact that this pro-inflammatory cytokine is mainly released by T and natural killer (NK) cells (Kulkarni et al., 2016), or as it can be released by macrophages only when they have previously been co-stimulated by both IL-12 and IL-18 (Darwich et al., 2009). Furthermore, the research carried out during this PhD shows that when U937-derived macrophages were stimulated by mCRP, these cells were not able to increase the release of IL-10 anti-inflammatory cytokines (**Fig. 4.10**). This result could indicate that mCRP has the potential to polarize macrophages towards an M1 pro-inflammatory phenotype (Trial et al., 2016), probably through the activation of the STAT-1/IRF-1/iNOS/NO intrinsic pathway, which was previously reported to be able to polarize several immune system cells towards an M1 pro-inflammatory phenotype (Jaruga et al., 2004; Lohoff and Mak, 2005; Xie et al., 2016). This hypothesis is not only supported by other previous studies, which confirm that mCRP is capable of generating NO (Mills et al., 2000; Mills, 2001; Sproston and Ashworth, 2018; Yao et al., 2019), but may also have some clinical value in pathologies where the accumulation of monocytes/macrophages and mCRP has been confirmed in the same tissue (Schwedler et al., 2003; Eisenhardt et al., 2009b; Slevin et al., 2015). Indeed, when this concomitant accumulation occurs, mCRP facilitates the macrophage polarization towards an M1 pro-inflammatory phenotype, this protein acerbates the inflammation by increasing the production of some pro-inflammatory cytokines (**Fig. 4.9**) and reduces the release of some growth factors and anti-inflammatory cytokines (King et al., 2014; Gilbert et al., 2016) which are specifically synthesized by the M2 anti-inflammatory phenotype (Liu

et al., 2018a; Krzyszczyk et al., 2018) where their presence may be compromised when mCRP binds with these immune system cells.

During the last few decades, several studies have reported that the mCRP pro-inflammatory activity is FCγR-mediated (Eisenhardt et al., 2009b; Okun et al., 2010; Thiele et al., 2014a; Thiele et al., 2015; Mkaddem et al., 2019). Knowing that these receptors are also expressed on macrophages (Satpathy et al., 2012; Chiba and Umegaki, 2013; Bain and Mowat, 2014; Vogelpoel et al., 2015), it was reasonable to study whether the mCRP-macrophage pro-inflammatory activity was mediated by the FCγRs. The results herein reported show that the FCγR antibodies did not increase the pro-inflammatory cytokine concentration on U937 macrophages (**Fig 4.11** and **Fig 4.12**). Furthermore, it did not provoke the phosphorylation MAPK or NF-KB signalling pathways (**Fig 4.13**). However, FCγR antibodies are unable to reduce mCRP pro-inflammatory activity in U937 monocyte-derived macrophages (**Fig 4.15**). The fact that the FCγR antibodies cannot limit mCRP might depend on the capacity mCRP has to bind with the lipid raft microdomain or cholesterol binding sequence (CBS), rather than on its binding to FCγRs (Ji et al., 2009; Jia et al., 2018).

The significance of understanding the involvement of FCγRs with mCRP pro-inflammatory activity was also linked to the potential biological effects that mCRP may have on other immune system cells, such as microglia. Microglia are tissue-resident macrophage cells that derive solely from the yolk-sac (Davies et al., 2013) and play a crucial role in brain inflammatory responses (Stephenson et al., 2018). It has recently been reported that microglia also express FCγRs (Fuller et al., 2014), which contributes substantially to microglia activation (Komine-Kobayashi et al., 2004) in Alzheimer's disease (AD) (Fuller et al., 2014).

Taking into consideration that two previous studies already confirmed that the presence of mCRP in the brain is associated to stroke-induced vascular dementia or AD (Slevin et al., 2015; Slevin et al., 2017), it would be interesting

to study whether the monomeric CRP forms could have a strong pro-inflammatory activity in this immune system cell.

6.3 Investigating the potential Acetylcholine anti-inflammatory activity on mCRP-stimulated monocyte-derivate macrophages.

After showing that macrophages are able to release pro-inflammatory cytokines when stimulated by mCRP, another key purpose of this project was to evaluate which type of inflammatory pathway the mCRP was able to trigger. Therefore, western blot experiments were carried out to determine which macrophage intrinsic pathways are triggered.

Recent studies have reported that when ECs are stimulated by mCRP, the activation of both MAPKs and NF-KB induces an increase in both the ICAM-2 and VCAM-1 release (Li et al., 2014; Yong and Cao, 2020). The results herein reported evidenced that both the MAPK (ERK1/2, JNK1/2, MAPKp38) and NF-KB intrinsic pathways were also activated in U937monocyte-derivated macrophages when these immune system cells were stimulated by mCRP at a concentration of 100 µg/mL (**Fig. 5.11**)**.** Knowing that these signalling pathway activities can be limitated by Nicotine and ACh (Yoshikawa et al., 2006a; Sun et al., 2013b), which would substantially reduce the pro-inflammatory cytokine release (Han et al., 2017; Jiang et al., 2017a; Delgado-Velez and Lasalde-Dominicci, 2018), it was assessed whether both ACh and Nicotine could be used as a potential SMI against mCRP pro-inflammatory activity, by binding with the U937 monocyte-derivate macrophages.

The data reported in chapter 5 evidenced that neither ACh nor Nicotine were cytotoxic to the U937-derived macrophages (**Fig. 5.2** and **Fig 5.3**) and did not trigger signalling pathways that could have been associated to a pro-inflammatory response (**Fig. 5.11**). Furthermore, when the macrophages were stimulated by these two SMIs, these immune system cells did not increase the pro-inflammatory cytokine secretion (**Fig. 5.4** and **Fig 5.5**) nor did they have other general anti-inflammatory proprieties (**Fig. 5.6** and **Fig. 5.7**) differently to what was reported in some studies (Yoshikawa et al., 2006a; Liu et al., 2015a).

However, even if neither ACh nor Nicotine were able to block the dissociation from nCRP into mCRP (**Fig. 3.4** and **Fig. 3.5**), the results herein reported showed that ACh was the only selected small molecular inhibitor able to reduce the mCRP induced increase in pro-inflammatory cytokines (**Fig. 5.9**). ACh reduced the concentration of both TNF-α and IL-6 cytokines, due to its ability to reduce the phosphorylation of the ERK/JNK/ NF-KB intrinsic pathway (**Fig. 5.11**).

As ACh is able to reduce IL-6 and TNF-α synthesis, down-regulating MAPK and NF-KB phosphorylation, there is the possibility that other MAPK and NF-KB small molecule inhibitors may also be useful in blocking or reducing the mCRP pro-inflammatory cytokine release. For example, Tectorigenin (TEC), a natural element derived from the *Belamcanda chinensis* (Zhang et al., 2017a), that has both anti-inflammatory and antioxidant proprieties (Wang et al., 2017), could reduce the inflammation activity by reducing the activation of the MAPK and NF-KB intrinsic pathways (Yang et al., 2012; Zeng et al., 2018). Indeed, Lim et al. (2018) also published evidence of its anti-inflammatory proprieties and reported that TEC is able to reduce the IL-6 and TNF-α synthesis, through the down-regulation of the ERK, JNK and NF-KB phosphorylation, which are the same intrinsic pathways reported in this study.

Resveratrol is another natural compound with anti-inflammatory activity and has been documented to be one of many strong bioactive polyphenol elements that can protect against CVD (Singh et al., 2015; Stephan et al., 2017) and stroke (Singh et al., 2013; Lopez et al., 2015). As aforementioned, ischemic stroke is a neurological medical condition where an accumulation of both mCRP and macrophages has been confirmed in the ischemic brain (Slevin et al., 2010; Chiba and Umegaki, 2013). An *in vivo* study reported that Resveratrol was able to reduce infarct size, ischemic injury, microglia activation and IL-1β, TNF-α and ROS concentration after a transient MCAO in both male and female C57BL/6 mice (Shin et al., 2010). Another study reported that Resveratrol was able to reduce both nCRP and triglyceride concentrations without any changes in weight, waist circumference or blood pressure (Bo et al., 2013). Later on, another two studies reported that, as Resveratrol reduced

ERK1/2, JNK and NF-KB phosphorylation and also decreased the TNF-α, IL-1β, IL-6 and NO synthesis and release (Shao et al., 2014a; Zhang et al., 2015). Therefore, taking into consideration the potential Resveratrol has to reduce nCRP levels, as well as the fact that its anti-inflammatory activity is similar to that of ACh, it can be hypothesized that this bioactive polyphenol compound may have a specific protective function against mCRP deleterious activity. Moreover, these two compounds may also exert some anti-inflammatory activities, without increasing NF-KB phosphorylation. This is what occurred when ACh was used alone in the experiments herein reported (**Fig. 5.11**) and this may be attributed to an activation of muscarinic acetylcholine receptors (mAChR), as previously reported by Profita et al. (2008).

mAChRs are widely distributed throughout human tissue (Caulfield and Birdsall, 1998) and are also expressed in both monocytes and macrophages (Kawashima et al., 2007) and allow ACh to exert its strong anti-inflammatory and antioxidant activity (Frinchi et al., 2019). However, in the last two decades, numerous studies have reported that the anti-inflammatory activity of ACh and Nicotine depends mainly on the capacity these two compounds have to specifically activate α7nAchR (Nathan, 2002; Tracey, 2002; Wang et al., 2003; de Jonge et al., 2005; Saeed et al., 2005; Pavlov and Tracey, 2005; Tracey, 2007; Ren et al., 2017; Han et al., 2017), which has also been reported to be strongly expressed on macrophages (Nathan, 2002; Tracey, 2002; Wang et al., 2003; de Jonge et al., 2005; Saeed et al., 2005; Tracey, 2007).

Differently to what has been reported in literature (Ren et al., 2017), i.e., that both ACh and Nicotine are able to bind to α7nAchR, the results obtained in this study evidenced that only ACh had anti-inflammatory activity against mCRP **(Fig. 5.9)**. Nicotine did not reduce pro-inflammatory cytokine secretion, maybe due to the fact that Nicotine itself increases nCRP expression when it binds to nAChR and activates the nAChR-MAPK signalling pathways on U937-monocyte-derived macrophages (Mao et al., 2012). This is confirmed by the activation of the MAPKp38 intrinsic pathway reported in **Fig 5.11,** which may also interfere with the anti-inflammatory activity of Nicotine against mCRP.

As ACh anti-inflammatory activity has been associated with α7nAchR activation, methyllycaconitine (MLA), a known selective α7nAchR antagonist (Carroll et al., 2007; Patel et al., 2017; Krafft et al., 2017; Zou et al., 2017), was used to assess whether ACh anti-inflammatory activity depends on the capacity of ACh to activate α7nAchR. The results evidenced that MLA does not induce any cytotoxic activity on U937-derived macrophages, either alone or in combination with SMIs (**Fig 5.15**), nor did MLA trigger any pro-inflammatory activity on U937 macrophages (**Fig 5.16**) or phosphorylate the MAPK or NF-KB signalling pathways (**Fig 5.18**). However, surprisingly, MLA did not reverse the ACh anti-inflammatory proprieties but rather increased them (**Fig 5.19**). These results are similar to other previous findings, which reported that MLA may play an anti-inflammatory role, as previously reported for microglia (Thomsen and Mikkelsen, 2012). A possible explanation for this could be that this α7nAchR antagonist does not increase the MAPKp38 or NF-KB intrinsic pathways (**Fig. 5.18**), differently to what has been evidenced when macrophages are stimulated by ACh or Nicotine alone (**Fig. 5.11**).

6.4 Conclusions.

A series of experiments were performed in this project to establish if and how monomeric-CRP might trigger or increase inflammation. The data obtained confirmed that the pentameric nCRP protein can be dissociated into a monomeric-CRP form (mCRP) and the use of two different cholinergic compounds, i.e., ACh and Nicotine does not block this dissociation. Furthermore, it was observed that macrophages up-regulate IL-1β and IL-6 gene expression when stimulated by mCRP at a concentration of 100 μg/mL. This up-regulation was also associated with an increase in the release of pro-inflammatory TNF-α and IL-6 cytokines through the activation of the ERK1/2, JNK1/2, MAPKp38 and NF-KB signalling pathways. The results also evidence that ACh plays a strong anti-inflammatory role against mCRP pro-inflammatory activity by working at both a pre- and post-transcriptional level (**Fig 6.1**).

In conclusion, the experiments carried out during this project evidenced that ACh, or other small molecule inhibitors with a similar structure, may be useful in blocking the mCRP pro-inflammatory activity that may be important in some chronic inflammatory medical conditions.

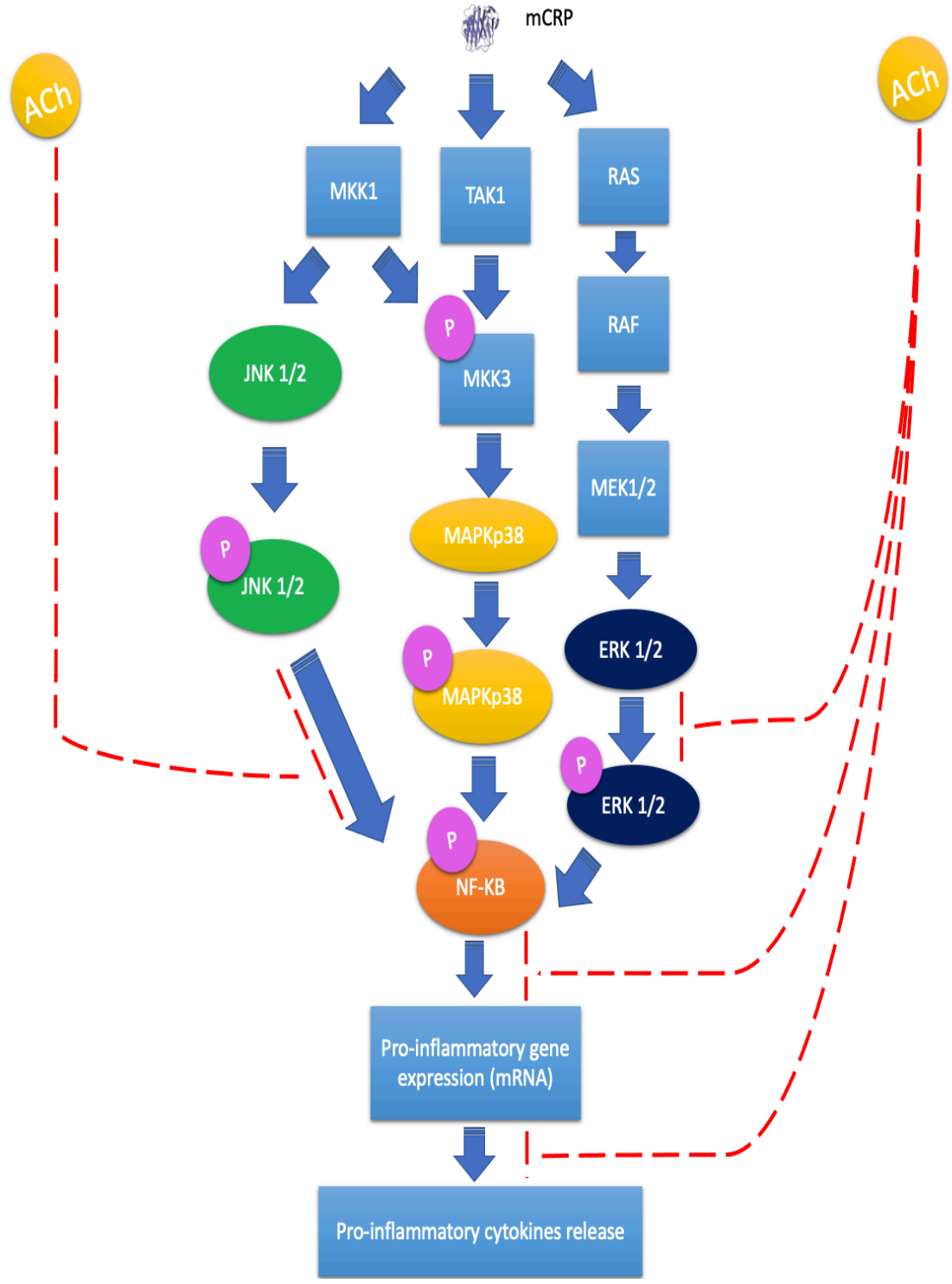


Fig 6.1. ACh decreases monomeric c-reactive protein pro-inflammatory activity. When monomeric-CRP activates the MAPKKK/MAPKs/MAPKs intrinsic pathways this leads to the nuclear translocation of the NF-KB. Transcription factor NF-KB increases the mRNA and protein expression of several pro-inflammatory cytokines. ACh is unable to block MAPKp38 phosphorylation, which promotes the NF-KB nuclear translocation associated with an increase of pro-inflammatory cytokine release. However, the use of ACh to reduce MAPKs (JNK1/2 and ERK1/2) and NF-KB phosphorylation, limits the pro-inflammatory cytokine release which is more elevated when macrophages are stimulated with mCRP alone. Furthermore, ACh may also be able to reduce the pro-inflammatory cytokine release by intervening at a post-transcriptional level, thus reducing the life of mRNA.

6.5 Limitations.

- Even if U937 cells have previously been used in other chronic inflammatory conditions, such diabetes, stroke, obesity and chronic kidney disease (Jain et al., 2002; Power et al., 2003; Yamagata et al., 2010; Fuggetta et al., 2019; Brito et al., 2020), none of these experiments provided evidence that the U937 monocytes, which differentiated into macrophages after PMA induction, behaved like primary circulating macrophages.

- As aforementioned, when monocytes differentiate into macrophages, they extravasate to the surrounding area and express specific surface markers, such as FCγR (Murray and Wynn, 2011; Satpathy et al., 2012). It has been reported that nCRP-mCRP dissociation led to an increase in monocyte adhesion and ROS, which is abolished when CD64 (FCγI), CD32 (FCγIIa), CD16 (FCγIII) receptors are blocked (Eisenhardt et al., 2009b). However, none of these experiments were aimed at assessing whether PMA could affect the expression of these receptors or whether antibodies were able to identify receptors on monocyte differentiated macrophages.

- A total of 24/30 the experiments were repeated 3 times. The 6 exceptions were optimization experiments which were carried out only once. This was because previous work in this group already had other similar complementary definitive results, i.e., the experiments reported in chapter 3.2 and 3.3 complemented the results obtained by the Surface plasmon resonance technique and published by Slevin et al. (2018)

- All the results herein reported are based on an *in-vitro* model. The main drawback of this experimental model is that it is challenging to obtain the same or similar results in an *in vivo* model.

6.5 Future Work.

Further studies could be carried out on the basis of the findings herein reported

- Previous studies reported that mCRP stimulation of ECs triggers the activation of both MAPKp38 and NF-KB signalling pathways (Li et al., 2014). Literature confirmed that when mCRP activates these signalling pathways there is an increase in the concentration of IL-6, ICAM-1, VCAM-1, which is associated to an elevated migration and recruitment of several immune system cells, such as monocytes, macrophages, natural killer cells and dendritic cells (Yang et al., 2008; Eisenhardt et al., 2009b; Yong and Cao, 2020). It has also been reported that ACh is able to bind with ECs (Shao et al., 2014b). Furthering these findings, a recent publication from this work, reported that ACh can limit (even if not significantly) the mCRP-mediated adhesion of EC to monocytes (Slevin et al., 2018). Future studies may be able to reach statistical significance, if ACh were used together with the SMI 6877002. Indeed, a recent study reported that when SMI 6877002 binds to monocytes it is able to: *(I)* reduce pro-inflammatory cytokine secretion; *(II)* increase the IL-10 anti-inflammatory cytokine release; *(III)* and reduce trans-endothelial monocyte migration (Aarts et al., 2017). Therefore, it is reasonable to presume that a combined stimulation with both ACh and SMI 6877002 may significantly reduce the mCRP pro-inflammatory activity in endothelial cell/macrophage co-cultures in *in-vitro* models. This could be useful in those pathologies were the endothelial, monocyte and mCRP interactions play a significant role in the development of pro-thrombotic and pro-inflammatory events (Molins et al., 2008; Eisenhardt et al., 2009b), which are often associated with medical conditions like arteriosclerosis or ischemic stroke (Badimon and Vilahur, 2014; Simons et al., 2015).

- ACh is able to reduce the mCRP pro-inflammatory activity, thus reducing ERK1/2, JNK1/2 and NF-kB phosphorylation. Knowing that both MAPK and NF-KB transcription factors regulate the expression of

genes involved in both acute and chronic inflammatory responses, it is may also be that other MAPKs and NF-KB SMIs could be used to block, or at least reduce, mCRP pro-inflammatory activity. Amongst the MAPK and NF-KB inhibitors, both Tectorigenin (TEC) and Resveratrol may be the most efficient candidates for this role due to their capacity to limited both MAPK and NF-KB activity.

- In the last few decades several studies have highlighted a number of common features in stroke and AD. (Desmond et al., 2002; Arbel-Ornath et al., 2013; Weinstein et al., 2013; Pase et al., 2017). After an ischemic stroke (IS) event, Howard (2016) advised that the first medical intervention after IS onset should be targeted towards restoring blood flow. However, the ischemia/reperfusion injury can exacerbate organ injury, allowing both nCRP and mCRP to penetrate the reperfused tissue (McFadyen et al., 2018). Other authors reported a strong link between this phenomenon and the increase of dementia incidence (Slevin et al., 2015; Slevin et al., 2017; Mammana et al., 2018), although the underlying mechanisms are still to be fully clarified. Based on the data presented herein, it would be interesting to study whether the binding of mCRP with microglia triggers a pro- inflammatory activity. Furthermore, it would also be of interest to assess whether the stimulation of ACh or Nicotine could be used as a potential therapeutically approach in microglia, based on fact that both of them have already been proposed as potential pharmacological treatment for several neurological diseases (Han et al., 2017).

REFERENCES.

Aarts, S., Seijkens, T. T. P., Kusters, P. J. H., van der Pol, S. M. A., Zarzycka, B., Heijnen, P., Beckers, L., den Toom, M., et al. (2017) 'Inhibition of CD40-TRAF6 interactions by the small molecule inhibitor 6877002 reduces neuroinflammation.' *J Neuroinflammation*, 14(1), May 12, 2017/05/13, p. 105.

Ågesen, F. N., Risgaard, B., Zachariasardóttir, S., Jabbari, R., Lynge, T. H., Ingemann-Hansen, O., Ottesen, G. L., Thomsen, J. L., et al. (2018) 'Sudden unexpected death caused by stroke: A nationwide study among children and young adults in Denmark.' *International journal of stroke : official journal of the International Stroke Society U6 -US U7 - Journal Article*, 13(3) p. 285.

Agnoli, C., Grioni, S., Pala, V., Allione, A., Matullo, G., Di Gaetano, C., Tagliabue, G., Sieri, S., et al. (2017) 'Biomarkers of inflammation and breast cancer risk: a case-control study nested in the EPIC-Varese cohort.' *SCIENTIFIC REPORTS*, 7 p. 1.

Agrawal, A., Cha-Molstad, H., Samols, D. and Kushner, I. (2003) 'Overexpressed nuclear factor-κB can participate in endogenous C-reactive protein induction, and enhances the effects of C/EBPβ and signal transducer and activator of transcription-3.' *Immunology*, 108(4) pp. 539-547.

Aguilera, G. and Liu, Y. (2012) 'The molecular physiology of CRH neurons.' *Front Neuroendocrinol*, 33(1), Jan, 2011/08/30, pp. 67-84.

Ahmed, Jadhav, A. B., Hassan, A. and Meng, Q. H. (2012) 'Acute phase reactants as novel predictors of cardiovascular disease.' *ISRN Inflamm*, 2012, May 6, 2012/01/01, p. 953461.

Ahmed, Shousha, W. G., El-Mezayen, H. A., Emara, I. A. and Hassan, M. E. (2020) 'New Biomarkers as Prognostic Factors for Cardiovascular Complications in Type 2 Diabetic Patients.' *Indian J Clin Biochem*, 35(1), Jan, 2020/02/20, pp. 54-62.

Ahn, Seger, R., Bratlien, R. L., Diltz, C. D., Tonks, N. K. and Krebs, E. G. (1991) 'Multiple components in an epidermal growth factor-stimulated protein kinase cascade. In vitro activation of a myelin basic protein/microtubule-associated protein 2 kinase.' *J Biol Chem*, 266(7), Mar 5, 1991/03/05, pp. 4220-4227.

Ai, Yan, J., Carrillo, E. and Ding, W. (2016) 'The Stress-Response MAP Kinase Signaling in Cardiac Arrhythmias.' *Rev Physiol Biochem Pharmacol*, 172 2016/11/17, pp. 77-100.

Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G. M., Cooper, N. R., Eikelenboom, P., et al. (2000) 'Inflammation and Alzheimer's disease.' *Neurobiol Aging*, 21(3), May-Jun, 2000/06/20, pp. 383-421.

Al-Nuaim, R. (1997) 'Effect of overweight and obesity on glucose intolerance and dyslipidemia in Saudi Arabia, epidemiological study.' *Diabetes Res Clin Pract*, 36(3), Jun, 1997/06/01, pp. 181-191.

Alam, F., Fatima, F., Orakzai, S., Iqbal, N. and Fatima, S. S. (2014) 'Elevated levels of ferritin and hs-CRP in type 2 diabetes.' *J Pak Med Assoc*, 64(12), Dec, 2015/04/07, pp. 1389-1391.

Albert, Bratz, I. N., Knudson, J. D., Rogers, P. A., Timmerman, J. M., Tune, J. D. and Dick, G. M. (2005) 'C-reactive protein does not relax vascular smooth muscle: effects mediated by

sodium azide in commercially available preparations.' *American Journal of Physiology - Heart and Circulatory Physiology*, 288(4) pp. 1786-1795.

Alkondon, M., Pereira, E. F. R., Cartes, W. S., Maelicke, A. and Albuquerque, E. X. (1997) 'Choline is a Selective Agonist of α7 Nicotinic Acetylcholine Receptors in the Rat Brain Neurons.' *European Journal of Neuroscience*, 9(12) pp. 2734-2742.

Allan, S. M., Tyrrell, P. J. and Rothwell, N. J. (2005) 'Interleukin-1 and neuronal injury.' *Nature Reviews Immunology*, 5(8) pp. 629-640.

Ambrose, J. A. and Barua, R. S. (2004) 'The pathophysiology of cigarette smoking and cardiovascular disease: an update.' *J Am Coll Cardiol*, 43(10), May 19, 2004/05/18, pp. 1731-1737.

Andersson, J. (2005) 'The inflammatory reflex--introduction.' *J Intern Med*, 257(2), Feb, 2005/01/20, pp. 122-125.

Arbel-Ornath, M., Hudry, E., Eikermann-Haerter, K., Hou, S., Gregory, J. L., Zhao, L., Betensky, R. A., Frosch, M. P., et al. (2013) 'Interstitial fluid drainage is impaired in ischemic stroke and Alzheimer's disease mouse models.' *Acta neuropathologica*, 126(3) p. 353.

Arenillas, J. F., Alvarez-Sabín, J., Molina, C. A., Chacón, P., Fernández-Cadenas, I., Ribó, M., Delgado, P., Rubiera, M., et al. (2008) 'Progression of symptomatic intracranial large artery atherosclerosis is associated with a proinflammatory state and impaired fibrinolysis.' *Stroke*, 39(5) pp. 1456-1463.

Aronson, D., Bartha, P., Zinder, O., Kerner, A., Markiewicz, W., Avizohar, O., Brook, G. J. and Levy, Y. (2004) 'Obesity is the major determinant of elevated C-reactive protein in subjects with the metabolic syndrome.' *Int J Obes Relat Metab Disord*, 28(5), May, 2004/03/03, pp. 674-679.

Atri, Guerfali, F. Z. and Laouini, D. (2018) 'Role of Human Macrophage Polarization in Inflammation during Infectious Diseases.' *Int J Mol Sci*, 19(6), Jun 19, 2018/06/21,

Authier, Coste, A., Martinez, A., Ferron, G., Gladieff, L., Prat, M., Naour, A. L., Coulson, K., et al. (2020) 'Circulating CD14high CD16low intermediate blood monocytes as a biomarker of ascites immune status and ovarian cancer progression.' *Journal for immunotherapy of cancer U6 Journal Article*, 8(1)

Aveleira, C. A., Lin, C.-M., Abcouwer, S. F., Ambrósio, A. F. and Antonetti, D. A. (2010) 'TNF-α signals through PKCζ/NF-κB to alter the tight junction complex and increase retinal endothelial cell permeability.' *Diabetes (New York, N.Y.)*, 59(11) pp. 2872-2882.

Babic, T. and Browning, K. N. (2014) 'The role of vagal neurocircuits in the regulation of nausea and vomiting.' *European Journal of Pharmacology*, 722 pp. 38-47.

Badimon, L. and Vilahur, G. (2014) 'Thrombosis formation on atherosclerotic lesions and plaque rupture.' *J Intern Med*, 276(6), Dec, 2014/08/27, pp. 618-632.

Baez-Pagan, C. A., Delgado-Velez, M. and Lasalde-Dominicci, J. A. (2015) 'Activation of the Macrophage alpha7 Nicotinic Acetylcholine Receptor and Control of Inflammation.' *J Neuroimmune Pharmacol*, 10(3), Sep, 2015/04/15, pp. 468-476.

Bain, C. C. and Mowat, A. M. (2014) 'The monocyte-macrophage axis in the intestine.' *Cellular Immunology*, 291(1-2) pp. 41-48.

Bakhshayesh-Eghbali, Roudbary, S. A., Basir Jafari, S., Nabizadeh, S. P., Naderi-Asrami, N. and Sohrabnejad, R. (2016) 'Ability of serum C-reactive protein and white blood cell cout in predicting acute schemic stroke. A short -term follow-up study.' *Caspian J Intern Med*, 7(3), Summer, 2016/10/21, pp. 206-210.

Balla, S., Nusair, M. B. and Alpert, M. A. (2013) 'Risk factors for atherosclerosis in patients with chronic kidney disease: recognition and management.' *Curr Opin Pharmacol*, 13(2), Apr, 2013/01/08, pp. 192-199.

Ballou, S. P. and Lozanski, G. (1992) 'Induction of inflammatory cytokine release from cultured human monocytes by C-reactive protein.' *Cytokine*, 4(5), Sep, 1992/09/01, pp. 361-368.

Banerjee, C. and Chimowitz, M. I. (2017) 'Stroke Caused by Atherosclerosis of the Major Intracranial Arteries.' *Circ Res*, 120(3), Feb 3, 2017/02/06, pp. 502-513.

Bardwell, L. (2006) 'Mechanisms of MAPK signalling specificity.' *Biochem Soc Trans*, 34(Pt 5), Nov, 2006/10/21, pp. 837-841.

Barnes, T. C., Anderson, M. E. and Moots, R. J. (2011) 'The many faces of interleukin-6: the role of IL-6 in inflammation, vasculopathy, and fibrosis in systemic sclerosis.' *Int J Rheumatol*, 2011 2011/09/24, p. 721608.

Barrett, T. J. (2020) 'Macrophages in Atherosclerosis Regression.' *Arterioscler Thromb Vasc Biol*, 40(1), Jan, 2019/11/15, pp. 20-33.

Bature, F., Guinn, B.-A., Pang, D. and Pappas, Y. (2017) 'Signs and symptoms preceding the diagnosis of Alzheimer's disease: a systematic scoping review of literature from 1937 to 2016.' *BMJ open U6 -US U7 - Journal Article*, 7(8) p. e015746.

Baud, V. and Karin, M. (2001) 'Signal transduction by tumor necrosis factor and its relatives.' *Trends Cell Biol*, 11(9), Sep, 2001/08/22, pp. 372-377.

Benowitz, N. L. (2009) 'Pharmacology of nicotine: addiction, smoking-induced disease, and therapeutics.' *Annu Rev Pharmacol Toxicol*, 49 2008/10/07, pp. 57-71.

Berg, K. E., Ljungcrantz, I., Andersson, L., Bryngelsson, C., Hedblad, B., Fredrikson, G. N., Nilsson, J. and Björkbacka, H. (2012) 'Elevated CD14++CD16-monocytes predict cardiovascular events.' *Circulation: Cardiovascular Genetics*, 5(1) pp. 122-131.

Bernik, T. R., Friedman, S. G., Ochani, M., DiRaimo, R., Ulloa, L., Yang, H., Sudan, S., Czura, C. J., et al. (2002) 'Pharmacological stimulation of the cholinergic antiinflammatory pathway.' *The Journal of experimental medicine*, 195(6) p. 781.

Berthoud, H. R. and Neuhuber, W. L. (2000) 'Functional and chemical anatomy of the afferent vagal system.' *Autonomic neuroscience : basic & clinical*, 85(1-3) pp. 1-17.

Bertoni, A. G., Burke, G. L., Owusu, J. A., Carnethon, M. R., Vaidya, D., Barr, R. G., Jenny, N. S., Ouyang, P., et al. (2010) 'Inflammation and the incidence of type 2 diabetes: the Multi-Ethnic Study of Atherosclerosis (MESA).' *Diabetes Care*, 33(4), Apr, 2010/01/26, pp. 804-810.

Béténé A Dooko, C., De Wit, S., Neuhaus, J., Palfreeman, A., Pepe, R., Pankow, J. S., Neaton, J. D., Groups, E. S., et al. (2014) 'Interleukin-6, high sensitivity C-reactive protein, and the development of type 2 diabetes among HIV-positive patients taking antiretroviral therapy.' *Journal of acquired immune deficiency syndromes (1999) U6 - US U7 - Journal Article*, 67(5) p. 538.

Bharadwaj, D., Stein, M.-P., Volzer, M., Mold, C. and Du Clos, T. W. (1999) 'The major receptor for C-reactive protein on leukocytes is Fcγ receptor II.' *Journal of Experimental Medicine*, 190(4) pp. 585-590.

Black, Kushner, I. and Samols, D. (2004) 'C-reactive Protein.' *Journal of Biological Chemistry*, 279(47) pp. 48487-48490.

Black, B., A. M., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., et al. (1997) 'A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells.' *Nature (London)*, 385(6618) p. 729.

Blank, J. L., Gerwins, P., Elliott, E. M., Sather, S. and Johnson, G. L. (1996) 'Molecular cloning of mitogen-activated protein/ERK kinase kinases (MEKK) 2 and 3. Regulation of sequential phosphorylation pathways involving mitogen-activated protein kinase and c-Jun kinase.' *J Biol Chem*, 271(10), Mar 8, 1996/03/08, pp. 5361-5368.

Bo, S., Ciccone, G., Castiglione, A., Gambino, R., De Michieli, F., Villois, P., Durazzo, M., Cavallo-Perin, P., et al. (2013) 'Anti-inflammatory and antioxidant effects of resveratrol in healthy smokers a randomized, double-blind, placebo-controlled, cross-over trial.' *Current Medicinal Chemistry*, 20(10) pp. 1323-1331.

Bobryshev, Ivanova, E. A., Chistiakov, D. A., Nikiforov, N. G. and Orekhov, A. N. (2016) 'Macrophages and Their Role in Atherosclerosis: Pathophysiology and Transcriptome Analysis.' *BioMed research international*, 2016 pp. 1-13.

Bogoyevitch, M. A. and Kobe, B. (2006) 'Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases.' *Microbiol Mol Biol Rev*, 70(4), Dec, 2006/12/13, pp. 1061-1095.

Bokhari, F. A. h., Shakoori, T. A., Butt, A. and Ghafoor, F. (2014) 'TNF-alpha: a risk factor for ischemic stroke.' *Journal of Ayub Medical College, Abbottabad : JAMC*, 26(2) pp. 111-114.

Boncler and Watala, C. (2009) 'Regulation of cell function by isoforms of C-reactive protein: a comparative analysis.' *Acta Biochim Pol*, 56(1) 2009/02/17, pp. 17-31.

Bonner, T. I. (1989) 'The molecular basis of muscarinic receptor diversity.' *Trends Neurosci*, 12(4), Apr, 1989/04/01, pp. 148-151.

Boraei, A. T. A., Singh, P. K., Sechi, M. and Satta, S. (2019) 'Discovery of novel functionalized 1,2,4-triazoles as PARP-1 inhibitors in breast cancer: Design, synthesis and antitumor activity evaluation.' *Eur J Med Chem*, 182, Nov 15, 2019/08/24, p. 111621.

Boras, E., Slevin, M., Alexander, M. Y., Aljohi, A., Gilmore, W., Ashworth, J., Krupinski, J., Potempa, L. A., et al. (2014) 'Monomeric C-reactive protein and Notch-3 co-operatively increase angiogenesis through PI3K signalling pathway.' *Cytokine*, 69(2), Oct, 2014/06/28, pp. 165-179.

Borovikova, L. V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G. I., Watkins, L. R., Wang, H., Abumrad, N., et al. (2000) 'Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin.' *Nature*, 405(6785) pp. 458-462.

Bowe, B., Xie, Y., Xian, H., Li, T. and Al-Aly, Z. (2017) 'Association between Monocyte Count and Risk of Incident CKD and Progression to ESRD.' *Clinical journal of the American Society of Nephrology*, 12(4) pp. 603-613.

Boyette, L. B., Macedo, C., Hadi, K., Elinoff, B. D., Walters, J. T., Ramaswamil, B., Chalasani, G., Taboas, J. M., et al. (2017) 'Phenotype, function, and differentiation potential of human monocyte subsets.' *PLOS ONE*, 12(4) p. e0176460.

Brancho, D., Tanaka, N., Jaeschke, A., Ventura, J. J., Kelkar, N., Tanaka, Y., Kyuuma, M., Takeshita, T., et al. (2003) 'Mechanism of p38 MAP kinase activation in vivo.' *Genes Dev*, 17(16), Aug 15, 2003/08/02, pp. 1969-1978.

Breit, S., Kupferberg, A., Rogler, G. and Hasler, G. (2018) 'Vagus Nerve as Modulator of the Brain-Gut Axis in Psychiatric and Inflammatory Disorders.' *FRONTIERS IN PSYCHIATRY*, 9 p. 44.

Brito, R. B. O., Rebello, J. F., Grabulosa, C. C., Pinto, W., Morales, A., Jr., Elias, R. M., Moyses, R. M. A. and Dalboni, M. A. (2020) '25-vitamin D reduces inflammation in uremic environment.' *Sci Rep*, 10(1), Jan 10, 2020/01/12, p. 128.

Burke, A. P., Tracy, R. P., Kolodgie, F., Malcom, G. T., Zieske, A., Kutys, R., Pestaner, J., Smialek, J., et al. (2002) 'Elevated C-reactive protein values and atherosclerosis in sudden coronary death: association with different pathologies.' *Circulation*, 105(17), Apr 30, 2002/05/01, pp. 2019-2023.

Buscà, R., Pouysségur, J. and Lenormand, P. (2016) 'ERK1 and ERK2 Map Kinases: Specific Roles or Functional Redundancy?' *Frontiers in cell and developmental biology*, 4 p. 53.

Businaro, R., Ippoliti, F., Ricci, S., Canitano, N. and Fuso, A. (2012) 'Alzheimer's disease promotion by obesity: induced mechanisms-molecular links and perspectives.' *Curr Gerontol Geriatr Res*, 2012 2012/06/16, p. 986823.

Calabro, P., Willerson, J. T. and Yeh, E. T. (2003) 'Inflammatory cytokines stimulated C-reactive protein production by human coronary artery smooth muscle cells.' *Circulation*, 108(16), Oct 21, 2003/10/08, pp. 1930-1932.

Camps, J. and García-Heredia, A. (2014) 'Introduction: oxidation and inflammation, a molecular link between non-communicable diseases.' *Adv Exp Med Biol*, 824 2014/07/21, pp. 1-4.

Canedo-Dorantes, L. and Canedo-Ayala, M. (2019) 'Skin Acute Wound Healing: A Comprehensive Review.' *Int J Inflam*, 2019 2019/07/06, p. 3706315.

Caprio, V., Badimon, L., Di Napoli, M., Fang, W.-H., Ferris, G. R., Guo, B., Iemma, R. S., Liu, D., et al. (2018) 'pCRP-mCRP Dissociation Mechanisms as Potential Targets for the Development of Small-Molecule Anti-Inflammatory Chemotherapeutics.' *Frontiers in immunology*, 9 p. 1089.

Caravaca, A. S., Centa, M., Gallina, A. L., Tarnawski, L. and Olofsson, P. S. (2020) 'Neural reflex control of vascular inflammation.' *Bioelectron Med*, 6 2020/04/02, p. 3.

Cargnello and Roux, P. P. (2011) 'Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases.' *Microbiol Mol Biol Rev*, 75(1), Mar, 2011/03/05, pp. 50-83.

Carroll, Ma, W., Navarro, H. A., Abraham, P., Wolckenhauer, S. A., Damaj, M. I. and Martin, B. R. (2007) 'Synthesis, nicotinic acetylcholine receptor binding, antinociceptive and seizure properties of methyllycaconitine analogs.' *Bioorg Med Chem*, 15(2), Jan 15, 2006/11/14, pp. 678-685.

Carter, J. and Lippa, C. F. (2001) 'Beta-amyloid, neuronal death and Alzheimer's disease.' *Curr Mol Med*, 1(6), Dec, 2002/03/20, pp. 733-737.

Caulfield, M. P. and Birdsall, N. J. (1998) 'International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors.' *Pharmacol Rev*, 50(2), Jun, 1998/07/02, pp. 279-290.

Caunt, C. J., Sale, M. J., Smith, P. D. and Cook, S. J. (2015) 'MEK1 and MEK2 inhibitors and cancer therapy: the long and winding road.' *Nat Rev Cancer*, 15(10), Oct, 2015/09/25, pp. 577-592.

Čejková, Králová-Lesná, I. and Poledne, R. (2016) 'Monocyte adhesion to the endothelium is an initial stage of atherosclerosis development.' *Cor et vasa (English ed.)*, 58(4) pp. e419-e425.

Ceron, J. J., Eckersall, P. D. and Martynez-Subiela, S. (2005) 'Acute phase proteins in dogs and cats: current knowledge and future perspectives.' *Vet Clin Pathol*, 34(2), Jun, 2005/05/20, pp. 85-99.

Chadee, D. N. and Kyriakis, J. M. (2010) 'Activation of SAPK/JNKs in vitro.' *Methods Mol Biol*, 661 2010/09/03, pp. 59-73.

Chaplin. (2010) 'Overview of the immune response.' *J Allergy Clin Immunol*, 125(2 Suppl 2), Feb, 2010/03/05, pp. S3-23.

Chapman, C. M. L., Beilby, J. P., McQuillan, B. M., Thompson, P. L. and Hung, J. (2004) 'Monocyte count, but not C-reactive protein or interleukin-6, is an independent risk marker for subclinical carotid atherosclerosis.' *Stroke*, 35(7) pp. 1619-1624.

Chen and Greene. (2004) 'Shaping the nuclear action of NF-κB.' *Nature Reviews Molecular Cell Biology*, 5(5) pp. 392-401.

Chen, Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., et al. (2018) 'Inflammatory responses and inflammation-associated diseases in organs.' *Oncotarget*, 9(6), Jan 23, 2018/02/23, pp. 7204-7218.

Chernyavsky, A. I., Arredondo, J., Skok, M. and Grando, S. A. (2010) 'Auto/paracrine control of inflammatory cytokines by acetylcholine in macrophage-like U937 cells through nicotinic receptors.' *Int Immunopharmacol*, 10(3), Mar, 2009/12/17, pp. 308-315.

Chernykh, E. R., Shevela, E. Y., Starostina, N. M., Morozov, S. A., Davydova, M. N., Menyaeva, E. V. and Ostanin, A. A. (2016) 'Safety and Therapeutic Potential of M2 Macrophages in Stroke Treatment.' *Cell Transplantation*, 25(8) pp. 1461-1471.

Chi and Melendez, A. J. (2007) 'Role of cell adhesion molecules and immune-cell migration in the initiation, onset and development of atherosclerosis.' *Cell Adh Migr*, 1(4), Oct-Dec, 2007/10/01, pp. 171-175.

Chi, Li, Y., Stehno-Bittel, L., Gao, J., Morrison, D. C., Stechschulte, D. J. and Dileepan, K. N. (2001) 'Interleukin-6 production by endothelial cells via stimulation of protease-activated receptors is amplified by endotoxin and tumor necrosis factor-alpha.' *J Interferon Cytokine Res*, 21(4), Apr, 2001/05/22, pp. 231-240.

Chiba and Umegaki, K. (2013) 'Pivotal roles of monocytes/macrophages in stroke.' *Mediators of Inflammation*, 2013 pp. 1-10.

Chirco, K. R., Whitmore, S. S., Wang, K., Potempa, L. A., Halder, J. A., Stone, E. M., Tucker, B. A. and Mullins, R. F. (2016) 'Monomeric C-reactive protein and inflammation in age-related macular degeneration.' *J Pathol*, 240(2), Oct, 2016/07/05, pp. 173-183.

Chistiakov, Bobryshev, Y. V. and Orekhov, A. N. (2015) 'Changes in transcriptome of macrophages in atherosclerosis.' *J Cell Mol Med*, 19(6), Jun, 2015/05/15, pp. 1163-1173.

Chistiakov, Grechko, A. V., Myasoedova, V. A., Melnichenko, A. A. and Orekhov, A. N. (2018) 'The role of monocytosis and neutrophilia in atherosclerosis.' *Journal of Cellular and Molecular Medicine*, 22(3) pp. 1366-1382.

Choi, Joseph, L. and Pilote, L. (2013) 'Obesity and C-reactive protein in various populations: a systematic review and meta-analysis.' *Obesity reviews*, 14(3) pp. 232-244.

Choi, Kim, J. H., Lim, S., Lim, J. Y., Kim, K. W., Park, K. S. and Jang, H. C. (2017) 'Monocyte count as a predictor of cardiovascular mortality in older Korean people.' *Age and ageing*, 46(3) pp. 433-438.

Chow, A., Merad, M. and Brown, B. D. (2011) 'Studying the mononuclear phagocyte system in the molecular age.' *Nature Reviews Immunology*, 11(11) pp. 788-798.

Ciubotaru, I., Potempa, L. A. and Wander, R. C. (2005) 'Production of modified C-reactive protein in U937-derived macrophages.' *EXPERIMENTAL BIOLOGY AND MEDICINE*, 230(10) pp. 762-770.

Clark, A. R., Dean, J. L. E. and Saklatvala, J. (2009) 'The p38 MAPK pathway mediates both antiinflammatory and proinflammatory processes: Comment on the article by Damjanov and the editorial by Genovese.' *Arthritis & Rheumatism*, 60(11) pp. 3513-3514.

Cockcroft, V. B., Osguthorpe, D. J., Barnard, E. A., Friday, A. E. and Lunt, G. G. (1990) 'Ligand-gated ion channels. Homology and diversity.' *Mol Neurobiol*, 4(3-4), Fall-Winter, 1990/01/01, pp. 129-169.

Colberg, Sigal, R. J., Yardley, J. E., Riddell, M. C., Dunstan, D. W., Dempsey, P. C., Horton, E. S., Castorino, K., et al. (2016) 'Physical Activity/Exercise and Diabetes: A Position Statement of the American Diabetes Association.' *Diabetes Care*, 39(11), Nov, 2016/12/08, pp. 2065-2079.

Colberg, Albright, A. L., Blissmer, B. J., Braun, B., Chasan-Taber, L., Fernhall, B., Regensteiner, J. G., Rubin, R. R., et al. (2010) 'Exercise and type 2 diabetes: American College of Sports Medicine and the American Diabetes Association: joint position statement. Exercise and type 2 diabetes.' *Med Sci Sports Exerc*, 42(12), Dec, 2010/11/19, pp. 2282-2303.

Cossarizza, A., Chang, H.-D., Radbruch, A., Anä, I., Annunziato, F., Bacher, P., Barnaba, V., Battistini, L., et al. (2017) 'Guidelines for the use of flow cytometry and cell sorting in immunological studies.' *European journal of immunology*, 47(10) pp. 1584-1797.

Craig, Stevens, M. V., Vaillancourt, R. R. and Camenisch, T. D. (2008) 'MAP3Ks as central regulators of cell fate during development.' *Developmental dynamics*, 237(11) pp. 3102-3114.

Cray, C., Zaias, J. and Altman, N. H. (2009) 'Acute phase response in animals: a review.' *Comp Med*, 59(6), Dec, 2009/12/26, pp. 517-526.

Daniele, Guardado Mendoza, R., Winnier, D., Fiorentino, T. V., Pengou, Z., Cornell, J., Andreozzi, F., Jenkinson, C., et al. (2014) 'The inflammatory status score including IL-6, TNF-alpha, osteopontin, fractalkine, MCP-1 and adiponectin underlies whole-body insulin resistance and hyperglycemia in type 2 diabetes mellitus.' *Acta Diabetol*, 51(1), Feb, 2013/12/29, pp. 123-131.

Dantzer, R., Konsman, J. P., Bluthé, R. M. and Kelley, K. W. (2000) 'Neural and humoral pathways of communication from the immune system to the brain: parallel or convergent?' *Autonomic neuroscience : basic & clinical*, 85(1-3) pp. 60-65.

Darwich, L., Coma, G., Peña, R., Bellido, R., Blanco, E. J. J., Este, J. A., Borras, F. E., Clotet, B., et al. (2009) 'Secretion of interferon-γ by human macrophages demonstrated at the single-cell level after costimulation with interleukin (IL)-12 plus IL-18.' *Immunology*, 126(3) pp. 386-393.

Daub, H., Olsen, J. V., Bairlein, M., Gnad, F., Oppermann, F. S., Körner, R., Greff, Z., Kéri, G., et al. (2008) 'Kinase-Selective Enrichment Enables Quantitative Phosphoproteomics of the Kinome across the Cell Cycle.' *Molecular cell*, 31(3) pp. 438-448.

David, S. and Kroner, A. (2011) 'Repertoire of microglial and macrophage responses after spinal cord injury.' *Nature Reviews Neuroscience*, 12(7) pp. 388-399.

Davies, L. C., Jenkins, S. J., Allen, J. E. and Taylor, P. R. (2013) 'Tissue-resident macrophages.' *Nature immunology*, 14(10) pp. 986-995.

Davis. (2000) 'Signal Transduction by the JNK Group of MAP Kinases.' *Cell (Cambridge)*, 103(2) pp. 239-252.

Davis, R. J. (1994) 'MAPKs: new JNK expands the group.' *Trends Biochem Sci*, 19(11), Nov, 1994/11/01, pp. 470-473.

de Jonge, van der Zanden, E. P., The, F. O., Bijlsma, M. F., van Westerloo, D. J., Bennink, R. J., Berthoud, H.-R., Uematsu, S., et al. (2005) 'Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway.' *Nature immunology*, 6(8) pp. 844-851.

de Oliveira, de Campos Castilho, G. R., da Cunha, A. L., Miyajima, F. and de Oliveira Martins, D. T. (2017) 'Dilodendron bipinnatum Radlk. inhibits pro-inflammatory mediators through the induction of MKP-1 and the down-regulation of MAPKp38/JNK/NF-κB pathways and COX-2 in LPS-activated RAW 264.7 cells.' *J Ethnopharmacol*, 202, Apr 18, 2017/02/28, pp. 127-137.

de Rekeneire, N., Peila, R., Ding, J., Colbert, L. H., Visser, M., Shorr, R. I., Kritchevsky, S. B., Kuller, L. H., et al. (2006) 'Diabetes, hyperglycemia, and inflammation in older individuals: the health, aging and body composition study.' *Diabetes Care*, 29(8), Aug, 2006/07/29, pp. 1902-1908.

Del Reino, Alsina-Beauchamp, D., Escós, A., Cerezo-Guisado, M. I., Risco, A., Aparicio, N., Zur, R., Fernandez-Estévez, M., et al. (2014) 'Pro-oncogenic role of alternative p38 mitogen-activated protein kinases p38γ and p38δ, linking inflammation and cancer in colitis-associated colon cancer.' *Cancer Research*, 74(21) pp. 6150-6160.

Delgado-Velez, M. and Lasalde-Dominicci, J. A. (2018) 'The Cholinergic Anti-Inflammatory Response and the Role of Macrophages in HIV-Induced Inflammation.' *Int J Mol Sci*, 19(5), May 16, 2018/05/19,

Deming, D., Geiger, P., Chen, H., Vaccaro, A., Kunnimalaiyaan, M. and Holen, K. (2008) 'ZM336372, a Raf-1 activator, causes suppression of proliferation in a human hepatocellular carcinoma cell line.' *J Gastrointest Surg*, 12(5), May, 2008/02/27, pp. 852-857.

den Hertog, van Rossum, J. A., van der Worp, H. B., van Gemert, H. M., de Jonge, R., Koudstaal, P. J. and Dippel, D. W. (2009) 'C-reactive protein in the very early phase of acute ischemic stroke: association with poor outcome and death.' *J Neurol*, 256(12), Dec, 2009/07/18, pp. 2003-2008.

DePaoli, A. M. (2014) '20 years of leptin: leptin in common obesity and associated disorders of metabolism.' *J Endocrinol*, 223(1), Oct, 2014/06/29, pp. T71-81.

Deshmane, S. L., Kremlev, S., Amini, S. and Sawaya, B. E. (2009) 'Monocyte chemoattractant protein-1 (MCP-1): An overview.' *Journal of Interferon and Cytokine Research*, 29(6) pp. 313-325.

Desmond, Moroney, J. T., Sano, M. and Stern, Y. (2002) 'Incidence of dementia after ischemic stroke - Results of a longitudinal study.' *STROKE*, 33(9) pp. 2254-2260.

Devaraj and Jialal, I. (2011) 'C-Reactive Protein Polarizes Human Macrophages to an M1 Phenotype and Inhibits Transformation to the M2 Phenotype.' *Arteriosclerosis, Thrombosis, and Vascular Biology*, 31(6) pp. 1397-1402.

Devaraj, Kumaresan, P. R. and Jialal, I. (2004) 'Effect of C-reactive protein on chemokine expression in human aortic endothelial cells.' *J Mol Cell Cardiol*, 36(3), Mar, 2004/03/11, pp. 405-410.

Devaraj, Singh, U. and Jialal, I. (2009) 'The evolving role of C-reactive protein in atherothrombosis.' *Clin Chem*, 55(2), Feb, 2008/12/20, pp. 229-238.

Devevre, E. F., Renovato-Martins, M., Clement, K., Sautes-Fridman, C., Cremer, I. and Poitou, C. (2015) 'Profiling of the three circulating monocyte subpopulations in human obesity.' *J Immunol*, 194(8), Apr 15, 2015/03/20, pp. 3917-3923.

Di Napoli, Papa, F. and Bocola, V. (2001a) 'C-reactive protein in ischemic stroke: an independent prognostic factor.' *Stroke*, 32(4), Apr, 2001/04/03, pp. 917-924.

Di Napoli, Papa, F. and Bocola, V. (2001b) 'Prognostic influence of increased C-reactive protein and fibrinogen levels in ischemic stroke.' *Stroke*, 32(1), Jan, 2001/01/04, pp. 133-138.

Di Napoli, Parry-Jones, A. R., Smith, C. J., Hopkins, S. J., Slevin, M., Masotti, L., Campi, V., Singh, P., et al. (2014) 'C-Reactive Protein Predicts Hematoma Growth in Intracerebral Hemorrhage.' *STROKE*, 45(1) pp. 59-65.

Diehl, E. E., Haines, r. G. K., Radosevich, J. A. and Potempa, L. A. (2000) 'Immunohistochemical localization of modified C-reactive protein antigen in normal vascular tissue.' *The American journal of the medical sciences*, 319(2) p. 79.

Dinarello, C. A. (2009) 'Immunological and inflammatory functions of the interleukin-1 family.' *Annu Rev Immunol*, 27 2009/03/24, pp. 519-550.

Dinarello, C. A., Simon, A. and van der Meer, J. W. (2012) 'Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases.' *Nat Rev Drug Discov*, 11(8), Aug, 2012/08/02, pp. 633-652.

Dinh, K. M., Kaspersen, K. A., Mikkelsen, S., Pedersen, O. B., Petersen, M. S., Thørner, L. W., Hjalgrim, H., Rostgaard, K., et al. (2019) 'Low-grade inflammation is negatively associated with physical Health-Related Quality of Life in healthy individuals: Results from The Danish Blood Donor Study (DBDS).' *PloS one*, 14(3) p. e0214468.

Dixon, J. B., Hayden, M. J., Lambert, G. W., Dawood, T., Anderson, M. L., Dixon, M. E. and O'Brien, P. E. (2008) 'Raised CRP Levels in Obese Patients: Symptoms of Depression Have an Independent Positive Association.' *Obesity*, 16(9) pp. 2010-2015.

Dobolyi, A., Vincze, C., Pál, G. and Lovas, G. (2012) 'The neuroprotective functions of transforming growth factor beta proteins.' *International Journal of Molecular Sciences*, 13(7) pp. 8219-8258.

Dong, Davis, R. J. and Flavell, R. A. (2002) 'MAP KINASES IN THE IMMUNE RESPONSE.' *Annual review of immunology*, 20(1) pp. 55-72.

Dong, Nao, J. and Gao, Y. (2020) 'Peripheral Monocyte Count Predicts Outcomes in Patients with Acute Ischemic Stroke Treated with rtPA Thrombolysis.' *Neurotox Res*, 37(2), Feb, 2019/09/04, pp. 469-477.

Dörfel, M. J. and Huber, O. (2012) 'A phosphorylation hotspot within the occludin C-terminal domain.' *Annals of the New York Academy of Sciences*, 1257(1) pp. 38-44.

Du Clos and Mold, C. (2004) 'C-reactive protein: An activator of innate immunity and a modulator of adaptive immunity.' *Immunologic Research*, 30(3) pp. 261-277.

Dunand-Sauthier, I., Walker, C. A., Narasimhan, J., Pearce, A. K., Wek, R. C. and Humphrey, T. C. (2005) 'Stress-activated protein kinase pathway functions to support protein synthesis and translational adaptation in response to environmental stress in fission yeast.' *Eukaryot Cell*, 4(11), Nov, 2005/11/10, pp. 1785-1793.

Duris, K., Lipkova, J. and Jurajda, M. (2017) 'Cholinergic Anti-inflammatory Pathway and Stroke.' *Current drug delivery U6 -US U7 - Journal Article*, 14(4) p. 449.

Eder, K., Baffy, N., Falus, A. and Fulop, A. K. (2009) 'The major inflammatory mediator interleukin-6 and obesity.' *Inflamm Res*, 58(11), Nov, 2009/06/23, pp. 727-736.

Ediriweera, M. K., Tennekoon, K. H. and Samarakoon, S. R. (2018) 'In vitro assays and techniques utilized in anticancer drug discovery: In vitro assays in anticancer drug development.' *Journal of Applied Toxicology*,

Egawa, M., Mukai, K., Yoshikawa, S., Iki, M., Mukaida, N., Kawano, Y., Minegishi, Y. and Karasuyama, H. (2013) 'Inflammatory Monocytes Recruited to Allergic Skin Acquire an Anti-inflammatory M2 Phenotype via Basophil-Derived Interleukin-4.' *Immunity*, 38(3) pp. 570-580.

Eisenhardt, Thiele, J. R., Bannasch, H., Stark, G. B. and Peter, K. (2009a) 'C-reactive protein: How conformational changes influence inflammatory properties.' *Cell Cycle*, 8(23) pp. 3885-3892.

Eisenhardt, Habersberger, J., Oliva, K., Lancaster, G. I., Ayhan, M., Woollard, K. J., Bannasch, H., Rice, G. E., et al. (2011) 'A proteomic analysis of C-reactive protein stimulated THP-1 monocytes.' *Proteome Sci*, 9(1), Jan 10, 2011/01/12, p. 1.

Eisenhardt, Habersberger, J., Murphy, A., Chen, Y.-C., Woollard, K. J., Bassler, N., Qian, H., von zur Muhlen, C., et al. (2009b) 'Dissociation of Pentameric to Monomeric C-Reactive Protein on Activated Platelets Localizes Inflammation to Atherosclerotic Plaques.' *Circulation Research*, 105(2) pp. 128-137.

El Boukhrissi, F., Benbella, I., Ouleghzal, H., Safi, S., Bamou, Y. and Balouch, L. (2017) 'Evaluation of ultrasensitive CRP and microalbuminuria as cardiovascular risk markers in type 2 diabetic moroccan patients.' *Tunis Med*, 95(11), Novembre, 2017/01/01, pp. 982-987.

Eming, S. A., Krieg, T. and Davidson, J. M. (2007) 'Inflammation in wound repair: molecular and cellular mechanisms.' *J Invest Dermatol*, 127(3), Mar, 2007/02/15, pp. 514-525.

Engel, J. E. and Chade, A. R. (2019) 'Macrophage polarization in chronic kidney disease: a balancing act between renal recovery and decline?' *Am J Physiol Renal Physiol*, 317(6), Dec 1, 2019/10/01, pp. F1409-f1413.

Erta, Quintana, A. and Hidalgo, J. (2012) 'Interleukin-6, a major cytokine in the central nervous system.' *Int J Biol Sci*, 8(9) 2012/11/09, pp. 1254-1266.

Eskandari, F. and Sternberg, E. M. (2002) 'Neural-Immune Interactions in Health and Disease.' *Annals of the New York Academy of Sciences*, 966(1) pp. 20-27.

Eyers, C. E., McNeill, H., Knebel, A., Morrice, N., Arthur, S. J., Cuenda, A. and Cohen, P. (2005) 'The phosphorylation of CapZ-interacting protein (CapZIP) by stress-activated protein kinases triggers its dissociation from CapZ.' *Biochem J*, 389(Pt 1), Jul 1, 2005/04/27, pp. 127-135.

Fang, Y., Xu, C., Wu, P., Zhang, L. H., Li, D. W., Sun, J. H., Li, W. F. and Liao, Z. S. (2017) 'Prognostic role of C-reactive protein in patients with nasopharyngeal carcinoma: A meta-analysis and literature review.' *MEDICINE*, 96(45) p. e8463.

Fatkhullina, Peshkova, I. O. and Koltsova, E. K. (2016) 'The Role of Cytokines in the Development of Atherosclerosis.' *Biochemistry (Mosc)*, 81(11), Nov, 2016/12/05, pp. 1358-1370.

Feng, Y., Li, L. and Sun, X. H. (2011) 'Monocytes and Alzheimer's disease.' *Neurosci Bull*, 27(2), Apr, 2011/03/29, pp. 115-122.

Ferrarese, Mascarucci, P., Zoia, C., Cavarretta, R., Frigo, M., Begni, B., Sarinella, F., Frattola, L., et al. (1999) 'Increased cytokine release from peripheral blood cells after acute stroke.' *Journal of Cerebral Blood Flow and Metabolism*, 19(9) pp. 1004-1009.

Feuerstein, G. Z., Liu, T. and Barone, F. C. (1994) 'Cytokines, inflammation, and brain injury: Role of tumor necrosis factor-alpha.' *CEREBROVASC.BRAIN METAB.REV*, 6(4) pp. 341-360.

Flynn, M. C., Pernes, G., Lee, M. K. S., Nagareddy, P. R. and Murphy, A. J. (2019) 'Monocytes, Macrophages, and Metabolic Disease in Atherosclerosis.' *Front Pharmacol*, 10 2019/06/30, p. 666.

Fox, E. R., Benjamin, E. J., Sarpong, D. F., Nagarajarao, H., Taylor, J. K., Steffes, M. W., Salahudeen, A. K., Flessner, M. F., et al. (2010) 'The relation of C--reactive protein to chronic kidney disease in African Americans: the Jackson Heart Study.' *BMC Nephrol*, 11, Jan 15, 2010/01/19, p. 1.

Franceschi, C. and Campisi, J. (2014) 'Chronic Inflammation (Inflammaging) and Its Potential Contribution to Age-Associated Diseases.' *Journals of Gerontology Series A: Biomedical Sciences and Medical Sciences*, 69(Suppl_1) pp. S4-S9.

Freire and Dyke, V. (2013) 'Natural resolution of inflammation.' *Periodontology 2000*, 63(1) pp. 149-164.

Friedrich, K., Sommer, M., Strobel, S., Thrum, S., Bluher, M., Wagner, U. and Rossol, M. (2019) 'Perturbation of the Monocyte Compartment in Human Obesity.' *Front Immunol*, 10 2019/08/24, p. 1874.

Frinchi, M., Nuzzo, D., Scaduto, P., Di Carlo, M., Massenti, M. F., Belluardo, N. and Mudo, G. (2019) 'Anti-inflammatory and antioxidant effects of muscarinic acetylcholine receptor (mAChR) activation in the rat hippocampus.' *Sci Rep*, 9(1), Oct 2, 2019/10/04, p. 14233.

Fuggetta, M. P., Zonfrillo, M., Villiva, C., Bonmassar, E. and Ravagnan, G. (2019) 'Inflammatory Microenvironment and Adipogenic Differentiation in Obesity: The Inhibitory Effect of Theobromine in a Model of Human Obesity In Vitro.' *Mediators Inflamm*, 2019 2019/02/26, p. 1515621.

Fujii, T., Mashimo, M., Moriwaki, Y., Misawa, H., Ono, S., Horiguchi, K. and Kawashima, K. (2017) 'Expression and Function of the Cholinergic System in Immune Cells.' *Front Immunol*, 8 2017/09/22, p. 1085.

Fujita, M., Takada, Y. K., Izumiya, Y. and Takada, Y. (2014) 'The binding of monomeric C-reactive protein (mCRP) to Integrins αvβ3 and α4β1 is related to its pro-inflammatory action.' *PloS one*, 9(4) p. e93738.

Fuller, J. P., Stavenhagen, J. B. and Teeling, J. L. (2014) 'New roles for Fc receptors in neurodegeneration-the impact on Immunotherapy for Alzheimer's Disease.' *Frontiers in neuroscience*, 8(8) p. 235.

Gallowitsch-Puerta, M. and Pavlov, V. A. (2007) 'Neuro-immune interactions via the cholinergic anti-inflammatory pathway.' *Life Sciences*, 80(24-25) pp. 2325-2329.

Galván-Peña, S. and O'Neill, L. A. J. (2014) 'Metabolic reprograming in macrophage polarization.' *Frontiers in immunology*, 5 p. 420.

Galvis, Lips, K. S. and Kummer, W. (2006a) 'Expression of nicotinic acetylcholine receptors on murine alveolar macrophages.' *J Mol Neurosci*, 30(1-2) 2006/12/29, pp. 107-108.

Galvis, Lips, K. S. and Kummer, W. (2006b) 'Expression of nicotinic acetylcholine receptors on murine alveolar macrophages.' *Journal of Molecular Neuroscience*, 30(1) pp. 107-108.

Gans, S. L., Atema, J. J., Stoker, J., Toorenvliet, B. R., Laurell, H. and Boermeester, M. A. (2015) 'C-Reactive Protein and White Blood Cell Count as Triage Test Between Urgent and Nonurgent Conditions in 2961 Patients With Acute Abdominal Pain.' *Medicine (Baltimore)*, 94(9) p. e569.

Gao, J., Wang, A., Li, X., Li, J., Zhao, H., Zhang, J., Liang, J., Chen, S., et al. (2020) 'The Cumulative Exposure to High-Sensitivity C-Reactive Protein Predicts the Risk of Chronic Kidney Diseases.' *Kidney Blood Press Res*, 45(1) 2019/12/04, pp. 84-94.

Garlanda, C., Dinarello, C. A. and Mantovani, A. (2013) 'The interleukin-1 family: back to the future.' *Immunity*, 39(6), Dec 12, 2013/12/18, pp. 1003-1018.

Gawaz, M., Langer, H. and May, A. E. (2005) 'Platelets in inflammation and atherogenesis.' *J Clin Invest*, 115(12), Dec, 2005/12/03, pp. 3378-3384.

Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M. and Ley, K. (2010) 'Development of Monocytes, Macrophages, and Dendritic Cells.' *Science*, 327(5966) pp. 656-661.

Gelosa, P., Lecca, D., Fumagalli, M., Wypych, D., Pignieri, A., Cimino, M., Verderio, C., Enerbäck, M., et al. (2014) 'Microglia is a key player in the reduction of stroke damage promoted by the new antithrombotic agent ticagrelor.' *Journal of Cerebral Blood Flow and Metabolism*, 34(6) pp. 979-988.

Geng, H. H., Wang, Q., Li, B., Cui, B. B., Jin, Y. P., Fu, R. L., Zhang, Q., Wang, J. J., et al. (2017) 'Early neurological deterioration during the acute phase as a predictor of long-term outcome after first-ever ischemic stroke.' *MEDICINE*, 96(51) p. e9068.

Gerold, G., Zychlinsky, A., de Diego, J. L., Medicinska, f., Institutionen för klinisk, m., Virologi and Umeå, u. (2007) 'What is the role of Toll-like receptors in bacterial infections?' *Seminars in Immunology*, 19(1) pp. 41-47.

Gilbert, R. W. D., Vickaryous, M. K. and Viloria-Petit, A. M. (2016) 'Signalling by Transforming Growth Factor Beta Isoforms in Wound Healing and Tissue Regeneration.' *J Dev Biol*, 4(2), Jun 22, 2016/06/22,

Giraud, M., Cho, T. H., Nighoghossian, N., Maucort-Boulch, D., Deiana, G., Østergaard, L., Baron, J. C., Fiehler, J., et al. (2015) 'Early Blood Brain Barrier Changes in Acute Ischemic Stroke: A Sequential MRI Study.' *Journal of Neuroimaging*, 25(6) pp. 959-963.

Gkrania-Klotsas, Ye, Z., Cooper, A. J., Sharp, S. J., Luben, R., Biggs, M. L., Chen, L. K., Gokulakrishnan, K., et al. (2010) 'Differential white blood cell count and type 2 diabetes: systematic review and meta-analysis of cross-sectional and prospective studies.' *PLoS One*, 5(10), Oct 18, 2010/10/27, p. e13405.

Gliem, Schwaninger, M. and Jander, S. (2016) 'Protective features of peripheral monocytes/macrophages in stroke.' *BBA - Molecular Basis of Disease*, 1862(3) pp. 329-338.

Gliem, Mausberg, A. K., Lee, J. I., Simiantonakis, I., Rooijen, v. N., Hartung, H. P. and Jander, S. (2012) 'Macrophages prevent hemorrhagic infarct transformation in murine stroke models.' *Annals of Neurology*, 71(6) pp. 743-752.

Goehler, L. E., Gaykema, R. A., Nguyen, K. T., Lee, J. E., Tilders, F. J. H., Maier, S. F. and Watkins, L. N. (1999) 'Interleukin-1 beta in immune cells of the abdominal vagus nerve: a link between the immune and nervous system?' *Journal of Neuroscience*, 19(7) pp. 2799-2806.

Gómez and Cohen, P. (1991) 'Dissection of the protein kinase cascade by which nerve growth factor activates MAP kinases.' *Nature (London)*, 353(6340) p. 170.

Gomez, J. C., Yamada, M., Martin, J. R., Dang, H., Brickey, W. J., Bergmeier, W., Dinauer, M. C. and Doerschuk, C. M. (2015) 'Mechanisms of interferon-gamma production by neutrophils and its function during Streptococcus pneumoniae pneumonia.' *Am J Respir Cell Mol Biol*, 52(3), Mar, 2014/08/08, pp. 349-364.

Gonzalez, A. C. D. O., Andrade, Z. D. A., Costa, T. F. and Medrado, A. R. A. P. (2016) 'Wound healing - A literature review.' *Anais Brasileiros de Dermatologia*, 91(5) pp. 614-620.

Goodman, A. R., Cardozo, T., Abagyan, R., Altmeyer, A., Wisniewski, H. G. and Vilcek, J. (1996) 'Long pentraxins: an emerging group of proteins with diverse functions.' *Cytokine & growth factor reviews*, 7(2) pp. 191-202.

Gordon, S. (2003) 'Alternative activation of macrophages.' *Nature Reviews Immunology*, 3(1) pp. 23-35.

Gowayed, M. A., Rothe, K., Rossol, M., Attia, A. S., Wagner, U., Baerwald, C., El-Abhar, H. S. and Refaat, R. (2019) 'The role of α7nAChR in controlling the anti-inflammatory/anti-arthritic action of galantamine.' *Biochemical Pharmacology*, 170 p. 113665.

Gronberg, Johansen, F. F., Kristiansen, U. and Hasseldam, H. (2013) 'Leukocyte infiltration in experimental stroke.' *J Neuroinflammation*, 10, Sep 18, 2013/09/21, p. 115.

Gruys, E., Toussaint, M. J., Niewold, T. A. and Koopmans, S. J. (2005) 'Acute phase reaction and acute phase proteins.' *J Zhejiang Univ Sci B*, 6(11), Nov, 2005/10/28, pp. 1045-1056.

Guiteras, Flaquer, M. and Cruzado, J. M. (2016) 'Macrophage in chronic kidney disease.' *Clin Kidney J*, 9(6), Dec, 2016/12/21, pp. 765-771.

Gunasekaran, M. K., Viranaicken, W., Girard, A. C., Festy, F., Cesari, M., Roche, R. and Hoareau, L. (2013) 'Inflammation triggers high mobility group box 1 (HMGB1) secretion in adipose tissue, a potential link to obesity.' *Cytokine*, 64(1), Oct, 2013/08/14, pp. 103-111.

Guo, Y. J., Pan, W. W., Liu, S. B., Shen, Z. F., Xu, Y. and Hu, L. L. (2020) 'ERK/MAPK signalling pathway and tumorigenesis.' *Exp Ther Med*, 19(3), Mar, 2020/02/28, pp. 1997-2007.

Gupta, S. C., Kunnumakkara, A. B., Aggarwal, S. and Aggarwal, B. B. (2018) 'Inflammation, a Double-Edge Sword for Cancer and Other Age-Related Diseases.' *Front Immunol*, 9 2018/10/16, p. 2160.

Gurtner, G. C. W., Sabine, Barrandon, Y. and Longaker, M. T. (2008) 'Wound repair and regeneration.' *Nature*, 453(7193) pp. 314-321.

Guzmán-Ruiz, R., Ortega, F., Rodríguez, A., Vázquez-Martínez, R., Díaz-Ruiz, A., Garcia-Navarro, S., Giralt, M., Garcia-Rios, A., et al. (2014) 'Alarmin high-mobility group B1 (HMGB1) is regulated in human adipocytes in insulin resistance and influences insulin secretion in β-cells.' *International journal of obesity (2005)*, 38(12) pp. 1545-1554.

Hamdi, M., Kool, J., Cornelissen-Steijger, P., Carlotti, F., Popeijus, H. E., van der Burgt, C., Janssen, J. M., Yasui, A., et al. (2005) 'DNA damage in transcribed genes induces apoptosis via the JNK pathway and the JNK-phosphatase MKP-1.' *Oncogene*, 24(48), Nov 3, 2005/07/27, pp. 7135-7144.

Han and Molkentin, J. D. (2000) 'Regulation of MEF2 by p38 MAPK and its implication in cardiomyocyte biology.' *Trends Cardiovasc Med*, 10(1), Jan, 2001/01/11, pp. 19-22.

Han, Li, X. P. and Hao, J. W. (2017) 'The cholinergic anti-inflammatory pathway: An innovative treatment strategy for neurological diseases.' *NEUROSCIENCE AND BIOBEHAVIORAL REVIEWS*, 77 pp. 358-368.

Han, Shen, F. X., He, Y., Degos, V., Camus, M., Maze, M., Young, W. L. and Su, H. (2014) 'Activation of alpha-7 Nicotinic Acetylcholine Receptor Reduces Ischemic Stroke Injury through Reduction of Pro-Inflammatory Macrophages and Oxidative Stress.' *PLOS ONE*, 9(8) p. e105711.

Handley, M. E., Rasaiyaah, J., Chain, B. M. and Katz, D. R. (2007) 'Mixed lineage kinases (MLKs): a role in dendritic cells, inflammation and immunity?' *Int J Exp Pathol*, 88(2), Apr, 2007/04/06, pp. 111-126.

Hansson. (2005) 'Inflammation, atherosclerosis, and coronary artery disease.' *N Engl J Med*, 352(16), Apr 21, 2005/04/22, pp. 1685-1695.

Hansson and Hermansson, A. (2011) 'The immune system in atherosclerosis.' *Nat Immunol*, 12(3), Mar, 2011/02/16, pp. 204-212.

Harada, C., Nakamura, K., Namekata, K., Okumura, A., Mitamura, Y., Iizuka, Y., Kashiwagi, K., Yoshida, K., et al. (2006) 'Role of apoptosis signal-regulating kinase 1 in stress-induced neural cell apoptosis in vivo.' *Am J Pathol*, 168(1), Jan, 2006/01/10, pp. 261-269.

Haran, J. P., Beaudoin, F. L., Suner, S. and Lu, S. (2013) 'C-reactive protein as predictor of bacterial infection among patients with an influenza-like illness.' *Am J Emerg Med*, 31(1), Jan, 2012/09/05, pp. 137-144.

Hayashino, Y., Mashitani, T., Tsujii, S., Ishii, H., Diabetes, D. and Care Registry at Tenri Study, G. (2014) 'Elevated levels of hs-CRP are associated with high prevalence of depression in japanese patients with type 2 diabetes: the Diabetes Distress and Care Registry at Tenri (DDCRT 6).' *Diabetes care*, 37(9) pp. 2459-2465.

Hayden and Ghosh, S. (2008) 'Shared Principles in NF-κB Signaling.' *Cell*, 132(3) pp. 344-362.

Hayden, West, A. P. and Ghosh, S. (2006a) 'NF-κB and the immune response.' *Oncogene*, 25(51) pp. 6758-6780.

Hayden, West, A. P. and Ghosh, S. (2006b) 'NF- B and the immune response.' *Oncogene*, 25(51) pp. 6758-6780.

Heinrich, Malgorzata, Castell, J. V. and Andus, T. (1990) 'Interleukin-6 and the acute phase response.' *Biochem J*, 265(3), Feb 1, 1990/02/01, pp. 621-636.

Hemmi, H. and Akira, S. (2005) 'TLR signalling and the function of dendritic cells.' *Chem Immunol Allergy*, 86 2005/06/25, pp. 120-135.

Henel, G. and Schmitz, J. L. (2007) 'Basic theory and clinical applications of flow cytometry.' *LABMEDICINE*, 38(7) pp. 428-436.

Herold, T., Jurinovic, V., Arnreich, C., Lipworth, B. J., Hellmuth, J. C., von Bergwelt-Baildon, M., Klein, M. and Weinberger, T. (2020) 'Elevated levels of IL-6 and CRP predict the need for mechanical ventilation in COVID-19.' *J Allergy Clin Immunol*, 146(1), Jul, 2020/05/20, pp. 128-136 e124.

Hertog, H. M., Rossum, J. A., Worp, H. B., Gemert, H. M. A., Jonge, R., Koudstaal, P. J., Dippel, D. W. J., the, P. i., et al. (2009) 'C-reactive protein in the very early phase of acute

ischemic stroke: association with poor outcome and death.' *Journal of neurology*, 256(12) pp. 2003-2008.

Hida, A., Kawakami, A., Nakashima, T., Yamasaki, S., Sakai, H., Urayama, S., Ida, H., Nakamura, H., et al. (2000) 'Nuclear factor-kappaB and caspases co-operatively regulate the activation and apoptosis of human macrophages.' *Immunology*, 99(4) pp. 553-560.

Hirano, T., Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwamura, S., Nakajima, K., et al. (1986) 'Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin.' *Nature (London)*, 324(6092) p. 73.

Hitti, E., Iakovleva, T., Brook, M., Deppenmeier, S., Gruber, A. D., Radzioch, D., Clark, A. R., Blackshear, P. J., et al. (2006) 'Mitogen-Activated Protein Kinase-Activated Protein Kinase 2 Regulates Tumor Necrosis Factor mRNA Stability and Translation Mainly by Altering Tristetraprolin Expression, Stability, and Binding to Adenine/Uridine-Rich Element.' *Molecular and cellular biology*, 26(6) pp. 2399-2407.

Hoover. (2017) 'Cholinergic modulation of the immune system presents new approaches for treating inflammation.' *Pharmacol Ther*, 179, Nov, 2017/05/23, pp. 1-16.

Howard, R. S. (2016) 'The management of ischaemic stroke.' *Anaesthesia and Intensive Care Medicine*, 17(12) pp. 591-595.

Howland. (2014) 'Vagus Nerve Stimulation.' *Current Behavioral Neuroscience Reports*, 1(2) pp. 64-73.

Hsu, P. F., Pan, W. H., Yip, B. S., Chen, R. C. Y., Cheng, H. M. and Chuang, S. Y. (2017) 'C-Reactive Protein Predicts Incidence of Dementia in an Elderly Asian Community Cohort.' *JOURNAL OF THE AMERICAN MEDICAL DIRECTORS ASSOCIATION*, 18(3)

Hu, X., Li, P., Guo, Y., Wang, H., Leak, R. K., Chen, S., Gao, Y. and Chen, J. (2012) 'Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia.' *Stroke*, 43(11), Nov, 2012/08/31, pp. 3063-3070.

Hug, H., Mohajeri, M. H. and La Fata, G. (2018) 'Toll-Like Receptors: Regulators of the Immune Response in the Human Gut.' *Nutrients*, 10(2), Feb 13, 2018/02/14,

Hur, Lee, K. W. and Lee, H. J. (2004) 'Production of nitric oxide, tumor necrosis factor-α and interleukin-6 by RAW264.7 macrophage cells treated with lactic acid bacteria isolated from kimchi.' *BioFactors*, 21(1-4) pp. 123-125.

Hur, H. J., Lee, K. W. and Lee, H. J. (2004) 'Production of nitric oxide, tumor necrosis factor-α and interleukin-6 by RAW264.7 macrophage cells treated with lactic acid bacteria isolated from kimchi.' *BioFactors*, 21(1-4) pp. 123-125.

Hurlimann, J., Thorbecke, G. J. and Hochwald, G. M. (1966) 'The liver as the site of C-reactive protein formation.' *The Journal of experimental medicine*, 123(2) p. 365.

Hutchinson, W. L., Noble, G. E., Hawkins, P. N. and Pepys, M. B. (1994) 'The pentraxins, C-reactive protein and serum amyloid P component, are cleared and catabolized by hepatocytes in vivo.' *J Clin Invest*, 94(4), Oct, 1994/10/01, pp. 1390-1396.

Iadecola, C. (2013) 'The Pathobiology of Vascular Dementia.' *NEURON*, 80(4) pp. 844-866.

Iwamoto, N., Nishiyama, E., Ohwada, J. and Arai, H. (1994) 'Demonstration of CRP immunoreactivity in brains of Alzheimer's disease: immunohistochemical study using formic acid pretreatment of tissue sections.' *Neuroscience letters*, 177(1-2) p. 23.

Jain, Gautam, V. and Naseem, S. (2011) 'Acute-phase proteins: As diagnostic tool.' *J Pharm Bioallied Sci*, 3(1), Jan, 2011/03/25, pp. 118-127.

Jain, Kannan, K., Lim, G., McVie, R. and Joseph A. Bocchini, Jr. (2002) 'Hyperketonemia Increases Tumor Necrosis Factor-α Secretion in Cultured U937 Monocytes and Type 1 Diabetic Patients and Is Apparently Mediated by Oxidative Stress and cAMP Deficiency.' *Diabetes*, 51(7) p. 2287.

Jaruga, B., Hong, F., Kim, W.-H. and Gao, B. (2004) 'IFN-γ/STAT1 acts as a proinflammatory signal in T cell-mediated hepatitis via induction of multiple chemokines and adhesion molecules: a critical role of IRF-1.' *American Journal of Physiology - Gastrointestinal and Liver Physiology*, 287(5) pp. 1044-1052.

Jensen, L. E., Muzio, M., Mantovani, A. and Whitehead, A. S. (2000) 'IL-1 signaling cascade in liver cells and the involvement of a soluble form of the IL-1 receptor accessory protein.' *J Immunol*, 164(10), May 15, 2000/05/09, pp. 5277-5286.

Ji, Wu, Y., Zhu, L., Potempa, L. A., Sheng, F. L., Lu, W. and Zhao, J. (2007) 'Cell membranes and liposomes dissociate C-reactive protein (CRP) to form a new, biologically active structural intermediate: mCRP(m).' *FASEB J*, 21(1), Jan, 2006/11/23, pp. 284-294.

Ji, Ma, L., Bai, C.-J., Shi, J.-M., Li, H.-Y., Potempa, L. A., Filep, J. G., Zhao, J., et al. (2009) 'Monomeric C-reactive protein activates endothelial cells via interaction with lipid raft microdomains.' *FASEB Journal*, 23(6) pp. 1806-1816.

Jia, Z. K., Li, H. Y., Liang, Y. L., Potempa, L. A., Ji, S. R. and Wu, Y. (2018) 'Monomeric C-Reactive Protein Binds and Neutralizes Receptor Activator of NF-kappaB Ligand-Induced Osteoclast Differentiation.' *Front Immunol*, 9 2018/03/10, p. 234.

Jian, Z., Liu, R., Zhu, X., Smerin, D., Zhong, Y., Gu, L., Fang, W. and Xiong, X. (2019) 'The Involvement and Therapy Target of Immune Cells After Ischemic Stroke.' *Front Immunol*, 10 2019/10/02, p. 2167.

Jiang, Li, D., Han, R., Zhang, C., Jin, W. N., Wood, K., Liu, Q., Shi, F. D., et al. (2017a) 'Acetylcholine-producing NK cells attenuate CNS inflammation via modulation of infiltrating monocytes/macrophages.' *Proc Natl Acad Sci U S A*, 114(30), Jul 25, 2017/07/12, pp. E6202-E6211.

Jiang, Kong, W., Wang, Y., Ziai, W., Yang, Q., Zuo, F., Li, F., Wang, Y., et al. (2017b) 'Changes in the cellular immune system and circulating inflammatory markers of stroke patients.' *Oncotarget*, 8(2) pp. 3553-3567.

Jiang, Y., Gram, H., Zhao, M., New, L., Gu, J., Feng, L., Di Padova, F., Ulevitch, R. J., et al. (1997) 'Characterization of the structure and function of the fourth member of p38 group mitogen-activated protein kinases, p38delta.' *J Biol Chem*, 272(48), Nov 28, 1997/12/31, pp. 30122-30128.

Jiménez, M. C., Rexrode, K. M., Kotler, G., Everett, B. M., Glynn, R. J., Lee, I. M., Buring, J. E., Ridker, P. M., et al. (2016) 'Association Between Markers of Inflammation and Total Stroke by Hypertensive Status Among Women.' *American Journal of Hypertension*, 29(9) pp. 1117-1124.

Jin, Liu, L., Zhang, S., Nanda, A. and Li, G. (2013) 'Role of Inflammation and Its Mediators in Acute Ischemic Stroke.' *Journal of Cardiovascular Translational Research*, 6(5) pp. 834-851.

Johnsen, S. H., Fosse, E., Joakimsen, O., Mathiesen, E. B., Stensland-Bugge, E., Njølstad, I. and Arnesen, E. (2005) 'Monocyte count is a predictor of novel plaque formation: a 7-year follow-up study of 2610 persons without carotid plaque at baseline the Tromsø Study.' *Stroke (1970)*, 36(4) pp. 715-719.

Johnson and Lapadat, R. (2002) 'Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases.' *Science*, 298(5600), Dec 6, 2002/12/10, pp. 1911-1912.

Johnston and Webster, N. R. (2009) 'Cytokines and the immunomodulatory function of the vagus nerve.' *British Journal of Anaesthesia*, 102(4) pp. 453-462.

Johnston, B. and Conly, J. (2006) 'Tumour necrosis factor inhibitors and infection: What is there to know for infectious diseases physicians?' *The Canadian journal of infectious diseases & medical microbiology*, 17(4) pp. 209-212.

Kahn, S. E., Zinman, B., Haffner, S. M., O'Neill, M. C., Kravitz, B. G., Yu, D., Freed, M. I., Herman, W. H., et al. (2006) 'Obesity Is a Major Determinant of the Association of C-Reactive Protein Levels and the Metabolic Syndrome in Type 2 Diabetes.' *Diabetes*, 55(8) pp. 2357-2364.

Kaito, M., Araya, S., Gondo, Y., Fujita, M., Minato, N., Nakanishi, M. and Matsui, M. (2013) 'Relevance of distinct monocyte subsets to clinical course of ischemic stroke patients.' *PLoS One*, 8(8) 2013/08/13, p. e69409.

Kalra, R., Singh, S. P., Pena-Philippides, J. C., Langley, R. J., Razani-Boroujerdi, S. and Sopori, M. L. (2004) 'Immunosuppressive and anti-inflammatory effects of nicotine administered by patch in an animal model.' *Clin Diagn Lab Immunol*, 11(3), May, 2004/05/13, pp. 563-568.

Kaminska. (2005) 'MAPK signalling pathways as molecular targets for anti-inflammatory therapy--from molecular mechanisms to therapeutic benefits.' *Biochim Biophys Acta*, 1754(1-2), Dec 30, 2005/10/04, pp. 253-262.

Kanazawa, M., Ninomiya, I., Hatakeyama, M., Takahashi, T. and Shimohata, T. (2017) 'Microglia and Monocytes/Macrophages Polarization Reveal Novel Therapeutic Mechanism against Stroke.' *INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES*, 18(10) p. 2135.

Kang, Y. H. and Biswas, Subhra K. (2013) 'Basophil-Macrophage Dialog in Allergic Inflammation.' *Immunity*, 38(3) pp. 408-410.

Kany, S., Vollrath, J. T. and Relja, B. (2019) 'Cytokines in Inflammatory Disease.' *Int J Mol Sci*, 20(23), Nov 28, 2019/12/05,

Kapellos, T. S., Bonaguro, L., Gemund, I., Reusch, N., Saglam, A., Hinkley, E. R. and Schultze, J. L. (2019) 'Human Monocyte Subsets and Phenotypes in Major Chronic Inflammatory Diseases.' *Front Immunol*, 10 2019/09/24, p. 2035.

Karlin, A. (2002) 'Emerging structure of the nicotinic acetylcholine receptors.' *Nat Rev Neurosci*, 3(2), Feb, 2002/02/12, pp. 102-114.

Kasapis and Thompson, P. D. (2005) 'The effects of physical activity on serum C-reactive protein and inflammatory markers: A systematic review.' *Journal of the American College of Cardiology*, 45(10) pp. 1563-1569.

Katakami, N. (2018) 'Mechanism of Development of Atherosclerosis and Cardiovascular Disease in Diabetes Mellitus.' *J Atheroscler Thromb*, 25(1), Jan 1, 2017/10/03, pp. 27-39.

Katsuki, H. and Matsumoto, K. (2018) 'Nicotinic Acetylcholine Receptors in Regulation of Pathology of Cerebrovascular Disorders.' *In* Akaike, A., Shimohama, S. and Misu, Y. (eds.) *Nicotinic Acetylcholine Receptor Signaling in Neuroprotection*. Singaporepp. 113-136.

Kawasaki, T. and Kawai, T. (2014) 'Toll-like receptor signaling pathways.' *Front Immunol*, 5 2014/10/14, p. 461.

Kawashima, K., Yoshikawa, K., Fujii, Y. X., Moriwaki, Y. and Misawa, H. (2007) 'Expression and function of genes encoding cholinergic components in murine immune cells.' *Life Sci*, 80(24-25), May 30, 2007/03/27, pp. 2314-2319.

Kellar, K. J., Martino, A. M., Hall, D. P., Jr., Schwartz, R. D. and Taylor, R. L. (1985) 'High-affinity binding of [3H]acetylcholine to muscarinic cholinergic receptors.' *J Neurosci*, 5(6), Jun, 1985/06/01, pp. 1577-1582.

Khabar, K. S. (2014) 'Post-transcriptional control of cytokine gene expression in health and disease.' *J Interferon Cytokine Res*, 34(4), Apr, 2014/02/21, pp. 215-219.

Khalil, R. H. and Al-Humadi, N. (2020) 'Types of acute phase reactants and their importance in vaccination.' *Biomedical reports*, 12(4) pp. 143-152.

Khan, M. A., Schultz, S., Othman, A., Fleming, T., Lebrón-Galán, R., Rades, D., Clemente, D., Nawroth, P. P., et al. (2016) 'Hyperglycemia in stroke impairs polarization of monocytes/macrophages to a protective noninflammatory cell type.' *Journal of Neuroscience*, 36(36) pp. 9313-9325.

Khaodhiar, L., Ling, P. R., Blackburn, G. L. and Bistrian, B. R. (2004) 'Serum levels of interleukin-6 and C-reactive protein correlate with body mass index across the broad range of obesity.' *JPEN J Parenter Enteral Nutr*, 28(6), Nov-Dec, 2004/12/01, pp. 410-415.

Khreiss, Jozsef, L., Potempa, L. A. and Filep, J. G. (2004a) 'Opposing effects of C-reactive protein isoforms on shear-induced neutrophil-platelet adhesion and neutrophil aggregation in whole blood.' *Circulation*, 110(17), Oct 26, 2004/10/20, pp. 2713-2720.

Khreiss, József, L., Potempa, L. A. and Filep, J. n. G. (2004b) 'Conformational Rearrangement in C-Reactive Protein Is Required for Proinflammatory Actions on Human Endothelial Cells.' *Circulation (New York, N.Y.)*, 109(16) pp. 2016-2022.

Khreiss, T., József, L., Potempa, L. A. and Filep, J. G. (2004) 'Opposing effects of C-reactive protein isoforms on shear-induced neutrophil-platelet adhesion and neutrophil aggregation in whole blood.' *Circulation*, 110(17) pp. 2713-2720.

Khreiss, T., József, L., Hossain, S., John, S. D. C., Potempa, L. A. and Filep, J. G. (2002) 'Loss of Pentameric Symmetry of C-reactive Protein Is Associated with Delayed Apoptosis of Human Neutrophils.' *Journal of Biological Chemistry*, 277(43) pp. 40775-40781.

Kim and Cho, S. (2016) 'Microglia and Monocyte-Derived Macrophages in Stroke.' *Neurotherapeutics*, 13(4) pp. 702-718.

Kim, Lim, Park, Jang and Choi. (2017) 'Total and differential WBC counts are related with coronary artery atherosclerosis and increase the risk for cardiovascular disease in Koreans.' *PLoS One*, 12(7) 2017/07/29, p. e0180332.

King, A., Balaji, S., Le, L. D., Crombleholme, T. M. and Keswani, S. G. (2014) 'Regenerative Wound Healing: The Role of Interleukin-10.' *Adv Wound Care (New Rochelle)*, 3(4), Apr 1, 2014/04/24, pp. 315-323.

Klouche, M., Gottschling, S., Gerl, V., Hell, W., Husmann, M., Dorweiler, B., Messner, M. and Bhakdi, S. (1998) 'Atherogenic Properties of Enzymatically Degraded LDL: Selective Induction of MCP-1 and Cytotoxic Effects on Human Macrophages.' *Arteriosclerosis, Thrombosis, and Vascular Biology*, 18(9) pp. 1376-1385.

Koarai, A., Traves, S. L., Fenwick, P. S., Brown, S. M., Chana, K. K., Russell, R. E., Nicholson, A. G., Barnes, P. J., et al. (2012) 'Expression of muscarinic receptors by human macrophages.' *Eur Respir J*, 39(3), Mar, 2011/09/03, pp. 698-704.

Koh and DiPietro, L. A. (2011) 'Inflammation and wound healing: the role of the macrophage.' *Expert reviews in molecular medicine*, 13 p. e23.

Kolb and Zhang, W. (2020) 'Obesity and Breast Cancer: A Case of Inflamed Adipose Tissue.' *Cancers (Basel)*, 12(6), Jun 25, 2020/07/08,

Komine-Kobayashi, M., Chou, N., Mochizuki, H., Nakao, A., Mizuno, Y. and Urabe, T. (2004) 'Dual role of Fcgamma receptor in transient focal cerebral ischemia in mice.' *Stroke*, 35(4), Apr, 2004/02/28, pp. 958-963.

Kon, V., Linton, M. F. and Fazio, S. (2011) 'Atherosclerosis in chronic kidney disease: the role of macrophages.' *Nat Rev Nephrol*, 7(1), Jan, 2010/11/26, pp. 45-54.

Kovesdy, Furth, S. L. and Zoccali, C. (2017) 'Obesity and kidney disease: Hidden consequences of the epidemic.' *Afr J Prim Health Care Fam Med*, 9(1), Oct 26, 2017/11/09, pp. e1-e3.

Krafft, P. R., McBride, D., Rolland, W. B., Lekic, T., Flores, J. J. and Zhang, J. H. (2017) 'α7 Nicotinic Acetylcholine Receptor Stimulation Attenuates Neuroinflammation through JAK2-STAT3 Activation in Murine Models of Intracerebral Hemorrhage.' *BioMed Research International*, 2017 pp. 1-13.

Kramer, F., Torzewski, J., Kamenz, J., Veit, K., Hombach, V., Dedio, J. and Ivashchenko, Y. (2008) 'Interleukin-1β stimulates acute phase response and C-reactive protein synthesis by

inducing an NFκB- and C/EBPβ-dependent autocrine interleukin-6 loop.' *Molecular immunology*, 45(9) pp. 2678-2689.

Kravitz, B. A., Corrada, M. M. and Kawas, C. H. (2009) 'Elevated C-reactive protein levels are associated with prevalent dementia in the oldest-old.' *Alzheimer's & Dementia: The Journal of the Alzheimer's Association*, 5(4) pp. 318-323.

Krayem, I., Bazzi, S. and Karam, M. (2017) 'The combination of CRP isoforms with oxLDL decreases TNF-α and IL-6 release by U937-derived macrophages.' *Biomedical Reports*, 7(3) pp. 272-276.

Krzyszczyk, Schloss, R., Palmer, A. and Berthiaume, F. (2018) 'The Role of Macrophages in Acute and Chronic Wound Healing and Interventions to Promote Pro-wound Healing Phenotypes.' *FRONTIERS IN PHYSIOLOGY*, 9(MAY) p. 419.

Kuhlmann, C. R. W., Librizzi, L., Closhen, D., Pflanzner, T., Lessmann, V., Pietrzik, C. U., De Curtis, M. and Luhmann, H. J. (2009) 'Mechanisms of C-reactive protein-induced blood-brain barrier disruption.' *Stroke*, 40(4) pp. 1458-1466.

Kulkarni, A., Ganesan, P. and O'Donnell, L. A. (2016) 'Interferon Gamma: Influence on Neural Stem Cell Function in Neurodegenerative and Neuroinflammatory Disease.' *Clinical Medicine Insights: Pathology*, 2016(Suppl. 1) pp. 9-19.

Kullo, I. J., Hensrud, D. D. and Allison, T. G. (2002) 'Comparison of numbers of circulating blood monocytes in men grouped by body mass index (<25, 25 to <30, > or =30).' *Am J Cardiol*, 89(12), Jun 15, 2002/06/14, pp. 1441-1443.

Kushner and Feldmann, G. (1978) 'Control of the acute phase response. Demonstration of C-reactive protein synthesis and secretion by hepatocytes during acute inflammation in the rabbit.' *J Exp Med*, 148(2), Aug 1, 1978/08/01, pp. 466-477.

Kushner, Rzewnicki, D. and Samols, D. (2006) 'What Does Minor Elevation of C-Reactive Protein Signify?' *The American Journal of Medicine*, 119(2) pp. 166.e117-166.e128.

Kuta, A. E. and Baum, L. L. (1986) 'C-reactive protein is produced by a small number of normal human peripheral blood lymphocytes.' *The Journal of experimental medicine*, 164(1) p. 321.

Kyriakis and Avruch, J. (2001) 'Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation.' *Physiol Rev*, 81(2), Apr, 2001/03/29, pp. 807-869.

Kyriakis and Avruch, J. (2012) 'Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update.' *Physiol Rev*, 92(2), Apr, 2012/04/27, pp. 689-737.

Kyriakis, Force, T. L., Rapp, U. R., Bonventre, J. V. and Avruch, J. (1993) 'Mitogen regulation of c-Raf-1 protein kinase activity toward mitogen-activated protein kinase-kinase.' *J Biol Chem*, 268(21), Jul 25, 1993/07/25, pp. 16009-16019.

Kyriakis, App, H., Zhang, X. F., Banerjee, P., Brautigan, D. L., Rapp, U. R. and Avruch, J. (1992) 'Raf-1 activates MAP kinase-kinase.' *Nature*, 358(6385), Jul 30, 1992/07/30, pp. 417-421.

Lafontan. (2005) 'Fat cells: afferent and efferent messages define new approaches to treat obesity.' *Annual review of pharmacology and toxicology*, 45(1) pp. 119-146.

LaJevic, M. D., Suleiman, S., Cohen, R. L. and Chambers, D. A. (2011) 'Activation of p38 mitogen-activated protein kinase by norepinephrine in T-lineage cells.' *Immunology*, 132(2) pp. 197-208.

Lakhan, S. E. and Kirchgessner, A. (2011) 'Anti-inflammatory effects of nicotine in obesity and ulcerative colitis.' *J Transl Med*, 9, Aug 2, 2011/08/04, p. 129.

Lalramenga, P. C., Gupta, S. and P3, N. (2019) 'Study of C-Reactive Protein Significance in Chronic Kidney Disease.' *International Journal of Contemporary Medical Research* Volume 6 (Issue 6) pp. 2454-7379.

Landry, K. K., Alexander, K. S., Zakai, N. A., Judd, S. E., Kleindorfer, D. O., Howard, V. J., Howard, G. and Cushman, M. (2017) 'Association of stroke risk biomarkers with stroke symptoms: the Reasons for Geographic and Racial Differences in Stroke cohort.' *Journal of Thrombosis and Haemostasis*, 15(1) pp. 21-27.

Larouche, J., Sheoran, S., Maruyama, K. and Martino, M. M. (2018) 'Immune Regulation of Skin Wound Healing: Mechanisms and Novel Therapeutic Targets.' *Adv Wound Care (New Rochelle)*, 7(7), Jul 1, 2018/07/10, pp. 209-231.

Lawrence. (2009) 'The Nuclear Factor NF-kappa B Pathway in Inflammation.' *COLD SPRING HARBOR PERSPECTIVES IN BIOLOGY*, 1(6)

Lawrence and Gilroy, D. W. (2007) 'Chronic inflammation: a failure of resolution?' *Int J Exp Pathol*, 88(2), Apr, 2007/04/06, pp. 85-94.

Lawrence, Jivan, A., Shao, C., Duan, L., Goad, D., Zaganjor, E., Osborne, J., McGlynn, K., et al. (2008) 'The roles of MAPKs in disease.' *Cell Res*, 18(4), Apr, 2008/03/19, pp. 436-442.

Le Novere, N., Corringer, P. J. and Changeux, J. P. (2002) 'The diversity of subunit composition in nAChRs: evolutionary origins, physiologic and pharmacologic consequences.' *J Neurobiol*, 53(4), Dec, 2002/11/19, pp. 447-456.

Lee, Choe, J. W., Kim, H. K. and Sung, J. (2011) 'High-sensitivity C-reactive protein and cancer.' *J Epidemiol*, 21(3) 2011/03/04, pp. 161-168.

Lee, Cho, E., Kim, M. G., Jo, S. K., Cho, W. Y. and Kim, H. K. (2013) 'Proinflammatory CD14(+)CD16(+) monocytes are associated with vascular stiffness in predialysis patients with chronic kidney disease.' *Kidney Res Clin Pract*, 32(4), Dec, 2013/12/01, pp. 147-152.

Lega, I. C. and Lipscombe, L. L. (2020) 'Review: Diabetes, Obesity, and Cancer-Pathophysiology and Clinical Implications.' *Endocr Rev*, 41(1), Feb 1, 2019/11/14,

Leite, F., Leite, Â., Santos, A., Lima, M., Barbosa, J., Cosentino, M. and Ribeiro, L. (2017) 'Predictors of Subclinical Inflammatory Obesity: Plasma Levels of Leptin, Very Low-Density

Lipoprotein Cholesterol and CD14 Expression of CD16+ Monocytes.' *Obesity facts*, 10(4) pp. 308-322.

Lenormand, P., Sardet, C., Pagès, G., L'Allemain, G., Brunet, A. and Pouysségur, J. (1993) 'Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts.' *J Cell Biol*, 122(5), Sep, 1993/09/01, pp. 1079-1088.

Ley, K. (2017) 'M1 Means Kill; M2 Means Heal.' *Journal of immunology (Baltimore, Md. : 1950)*, 199(7) pp. 2191-2193.

Li, Hsieh, L. L., Tang, R. P., Liao, S. K. and Yeh, K. Y. (2009) 'Interleukin-6 (IL-6) released by macrophages induces IL-6 secretion in the human colon cancer HT-29 cell line.' *Hum Immunol*, 70(3), Mar, 2009/03/11, pp. 151-158.

Li, Qiao, W., Zhang, W., Li, F., Shi, J. and Dong, N. (2017) 'The shift of macrophages toward M1 phenotype promotes aortic valvular calcification.' *J Thorac Cardiovasc Surg*, 153(6), Jun, 2017/03/12, pp. 1318-1327 e1311.

Li, Fu, H., Tong, J., Li, Y. H., Qu, L. F., Wang, P. and Shen, F. M. (2018) 'Cholinergic anti-inflammatory pathway inhibits neointimal hyperplasia by suppressing inflammation and oxidative stress.' *Redox Biol*, 15, May, 2017/12/03, pp. 22-33.

Li, Wang, J., Wu, Y. X., Zhang, L., Liu, Z. P., Filep, J. G., Potempa, L. A., Wu, Y., et al. (2014) 'Topological localization of monomeric C-reactive protein determines proinflammatory endothelial cell responses.' *J Biol Chem*, 289(20), May 16, 2014/04/09, pp. 14283-14290.

Libby. (2002) 'Inflammation in atherosclerosis.' *Nature*, 420(6917), Dec 19-26, 2002/12/20, pp. 868-874.

Libby. (2012) 'History of Discovery: Inflammation in Atherosclerosis.' *Arteriosclerosis, thrombosis, and vascular biology*, 32(9) pp. 2045-2051.

Liberale, L., Montecucco, F., Bonaventura, A., Casetta, I., Seraceni, S., Trentini, A., Padroni, M., Dallegri, F., et al. (2017) 'Monocyte count at onset predicts poststroke outcomes during a 90-day follow-up.' *European journal of clinical investigation*, 47(10) pp. 702-710.

Liesz, A., Dalpke, A., Mracsko, E., Antoine, D. J., Roth, S., Zhou, W., Yang, H., Na, S.-Y., et al. (2015) 'DAMP signaling is a key pathway inducing immune modulation after brain injury.' *Journal of Neuroscience*, 35(2) pp. 583-598.

Lim, Kim, Y. J., Kim, B. Y., Park, G. and Jeong, S. J. (2018) 'The Anti-neuroinflammatory Activity of Tectorigenin Pretreatment via Downregulated NF-kappaB and ERK/JNK Pathways in BV-2 Microglial and Microglia Inactivation in Mice With Lipopolysaccharide.' *Front Pharmacol*, 9 2018/06/06, p. 462.

Lin, R., Liu, J., Gan, W. and Yang, G. (2004) 'C-reactive protein-induced expression of CD40-CD40L and the effect of lovastatin and fenofibrate on it in human vascular endothelial cells.' *Biol Pharm Bull*, 27(10), Oct, 2004/10/07, pp. 1537-1543.

Liu, Shepherd, E. G. and Nelin, L. D. (2007) 'MAPK phosphatases — regulating the immune response.' *Nature reviews. Immunology*, 7(3) pp. 202-212.

Liu, Zhang, L., Joo, D. and Sun, S.-C. (2017a) 'NF-κB signaling in inflammation.' *Signal Transduction and Targeted Therapy*, 2 p. 17023.

Liu, Kuang, W., Zhou, Q. and Zhang, Y. (2018a) 'TGF-beta1 secreted by M2 phenotype macrophages enhances the stemness and migration of glioma cells via the SMAD2/3 signalling pathway.' *Int J Mol Med*, 42(6), Dec, 2018/10/16, pp. 3395-3403.

Liu, Pan, C. E., Liu, Q. G., Yang, W. and Liu, X. M. (2003) 'Effect of NF-kappaB and p38 MAPK in activated monocytes/macrophages on pro-inflammatory cytokines of rats with acute pancreatitis.' *World J Gastroenterol*, 9(11), Nov, 2003/11/08, pp. 2513-2518.

Liu, Bui, T., Van Nguyen, H., Ong, B., Shen, Q. and Kamalasena, D. (2010) 'Serum C-reactive protein as a biomarker for early detection of bacterial infection in the older patient.' *Age and ageing*, 39(5) pp. 559-565.

Liu, Zhao, N., Li, D., Zeng, Z., Shao, Q., Peng, F., Wang, Y. and Qian, K. (2015a) 'Anti-inflammatory effect of acetylcholine on lipopolysaccharide induced inflammatory response of alveolar macrophages.' *Chinese Critical Care Medicine*, 27(10) pp. 811-815.

Liu, Zang, P., Guo, J. M., Wang, W., Dong, W. Z., Guo, W., Xiong, Z. G., Wang, W. Z., et al. (2012) 'Involvement of acetylcholine-alpha7nAChR in the protective effects of arterial baroreflex against ischemic stroke.' *CNS Neurosci Ther*, 18(11), Nov, 2012/10/31, pp. 918-926.

Liu, Zhang, C., Zhang, Z., Zhang, Z., Ji, W., Cao, S., Cai, X., Lei, D., et al. (2017b) 'E26 Transformation-Specific Transcription Factor ETS2 as an Oncogene Promotes the Progression of Hypopharyngeal Cancer.' *Cancer biotherapy & radiopharmaceuticals U6 -US U7 - Journal Article*, 32(9) p. 327.

Liu, Zhang, X., Tan, Q. L., Xu, W. X., Zhou, C. Y., Luo, M., Li, X., Huang, R. Y., et al. (2017c) 'NF-kappaB pathways are involved in M1 polarization of RAW 264.7 macrophage by polyporus polysaccharide in the tumor microenvironment.' *PLoS One*, 12(11) 2017/11/21, p. e0188317.

Liu, Liu, C., Jiang, L., Li, M., Long, T., He, W., Qin, G., Chen, L., et al. (2018b) 'alpha7 Nicotinic acetylcholine receptor-mediated anti-inflammatory effect in a chronic migraine rat model via the attenuation of glial cell activation.' *J Pain Res*, 11 2018/06/27, pp. 1129-1140.

Liu, Zeng, X. N., Hui, Y. J., Zhu, C. L., Wu, J., Taylor, D. H., Ji, J., Fan, W. M., et al. (2015b) 'Activation of alpha 7 nicotinic acetylcholine receptors protects astrocytes against oxidative stress-induced apoptosis: Implications for Parkinson's disease.' *NEUROPHARMACOLOGY*, 91 pp. 87-96.

Liu, Li, Y., Jiang, R., Nie, C., Zeng, Z., Zhao, N., Huang, C., Shao, Q., et al. (2015c) 'miR-132 inhibits lipopolysaccharide-induced inflammation in alveolar macrophages by the cholinergic anti-inflammatory pathway.' *Exp Lung Res*, 41(5), Jun, 2015/06/09, pp. 261-269.

Loffreda, S., Yang, S. Q., Lin, H. Z., Karp, C. L., Brengman, M. L., Wang, D. J., Klein, A. S., Bulkley, G. B., et al. (1998) 'Leptin regulates proinflammatory immune responses.' *Faseb j*, 12(1), Jan, 1998/01/23, pp. 57-65.

Lohoff, M. and Mak, T. W. (2005) 'Roles of interferon-regulatory factors in T-helper-cell differentiation.' *Nature reviews. Immunology*, 5(2) pp. 125-135.

Lombardo and Maskos, U. (2015) 'Role of the nicotinic acetylcholine receptor in Alzheimer's disease pathology and treatment.' *Neuropharmacology*, 96(Pt B) pp. 255-262.

Lopez, Dempsey, R. J. and Vemuganti, R. (2015) 'Resveratrol neuroprotection in stroke and traumatic CNS injury.' *NEUROCHEMISTRY INTERNATIONAL*, 89 pp. 75-82.

Lopez-Castejon, G. and Brough, D. (2011) 'Understanding the mechanism of IL-1beta secretion.' *Cytokine Growth Factor Rev*, 22(4), Aug, 2011/10/25, pp. 189-195.

Lowenstein, C. and Matsushita, K. (2004) 'The acute phase response and atherosclerosis.' *Drug Discovery Today: Disease Mechanisms*, 1(1) pp. 17-22.

Lu and Xu, S. (2006) 'ERK1/2 MAP kinases in cell survival and apoptosis.' *IUBMB Life*, 58(11), Nov, 2006/11/07, pp. 621-631.

Lu, Mold, C., Du Clos, T. W. and Sun, P. D. (2018) 'Pentraxins and Fc Receptor-Mediated Immune Responses.' *Front Immunol*, 9 2018/11/30, p. 2607.

Lu, Marjon, K. D., Mold, C., Du Clos, T. W. and Sun, P. D. (2012) 'Pentraxins and Fc receptors.' *Immunological Reviews*, 250(1) pp. 230-238.

Luan and Yao, Y.-M. (2018) 'The Clinical Significance and Potential Role of C-Reactive Protein in Chronic Inflammatory and Neurodegenerative Diseases.' *Frontiers in immunology*, 9 p. 1302.

Luna, J. M., Moon, Y. P., Liu, K. M., Spitalnik, S., Paik, M. C., Cheung, K., Sacco, R. L. and Elkind, M. S. (2014) 'High-sensitivity C-reactive protein and interleukin-6-dominant inflammation and ischemic stroke risk: the northern Manhattan study.' *Stroke*, 45(4), Apr, 2014/03/15, pp. 979-987.

Maeda, S. (2010) 'NF-kappaB, JNK, and TLR Signaling Pathways in Hepatocarcinogenesis.' *Gastroenterol Res Pract*, 2010 2010/12/15, p. 367694.

Mahmood, T. and Yang, P.-C. (2012) 'Western blot: Technique, theory, and trouble shooting.' *North American Journal of Medical Sciences*, 4(9) pp. 429-434.

Mahtani, K. R., Brook, M., Dean, J. L., Sully, G., Saklatvala, J. and Clark, A. R. (2001) 'Mitogen-activated protein kinase p38 controls the expression and posttranslational modification of tristetraprolin, a regulator of tumor necrosis factor alpha mRNA stability.' *Mol Cell Biol*, 21(19), Oct, 2001/09/05, pp. 6461-6469.

Maik-Rachline, Zehorai, E., Hanoch, T., Blenis, J. and Seger, R. (2018) 'The nuclear translocation of the kinases p38 and JNK promotes inflammation-induced cancer.' *Science Signaling*, 11(525)

Maizels, E. T., Mukherjee, A., Sithanandam, G., Peters, C. A., Cottom, J., Mayo, K. E. and Hunzicker-Dunn, M. (2001) 'Developmental regulation of mitogen-activated protein kinase-activated kinases-2 and -3 (MAPKAPK-2/-3) in vivo during corpus luteum formation in the rat.' *Mol Endocrinol*, 15(5), May, 2001/05/01, pp. 716-733.

Makki, K., Froguel, P. and Wolowczuk, I. (2013) 'Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines.' *ISRN Inflamm*, 2013, Dec 22, 2014/01/24, p. 139239.

Malin, Kullman, E. L., Scelsi, A. R., Haus, J. M., Filion, J., Pagadala, M. R., Godin, J. P., Kochhar, S., et al. (2018) 'A whole-grain diet reduces peripheral insulin resistance and improves glucose kinetics in obese adults: A randomized-controlled trial.' *Metabolism*, 82, May, 2018/01/07, pp. 111-117.

Malissen, B., Tamoutounour, S. and Henri, S. (2014) 'The origins and functions of dendritic cells and macrophages in the skin.' *Nature Reviews Immunology*, 14(6) pp. 417-428.

Mammana, S., Fagone, P., Cavalli, E., Basile, M. S., Petralia, M. C., Nicoletti, F., Bramanti, P. and Mazzon, E. (2018) 'The Role of Macrophages in Neuroinflammatory and Neurodegenerative Pathways of Alzheimer's Disease, Amyotrophic Lateral Sclerosis, and Multiple Sclerosis: Pathogenetic Cellular Effectors and Potential Therapeutic Targets.' *Int J Mol Sci*, 19(3), Mar 13, 2018/03/14,

Mandrekar, P. and Szabo, G. (2009) 'Signalling pathways in alcohol-induced liver inflammation.' *J Hepatol*, 50(6), Jun, 2009/04/29, pp. 1258-1266.

Mao, J. J., Liu, J. T., Pang, X. M., Li, M., Song, J. Y., Han, C. J., Wu, D. and Wang, S. Y. (2012) 'Nicotine induces the expression of C-reactive protein via MAPK-dependent signal pathway in U937 macrophages.' *MOLECULES AND CELLS*, 34(5) pp. 457-461.

Markanday, A. (2015) 'Acute Phase Reactants in Infections: Evidence-Based Review and a Guide for Clinicians.' *Open Forum Infect Dis*, 2(3), Sep, 2015/08/11, p. ofv098.

Martin, P. (1997) 'Wound Healing--Aiming for Perfect Skin Regeneration.' *Science*, 276(5309) pp. 75-81.

Martinez, P. F., Bonomo, C., Guizoni, D. M., Junior, S. A. O., Damatto, R. L., Cezar, M. D. M., Lima, A. R. R., Pagan, L. U., et al. (2016) 'Modulation of MAPK and NF-κB Signaling Pathways by Antioxidant Therapy in Skeletal Muscle of Heart Failure Rats.' *Cellular physiology and biochemistry*, 39(1) pp. 371-384.

Mathew, R. O., Bangalore, S., Lavelle, M. P., Pellikka, P. A., Sidhu, M. S., Boden, W. E. and Asif, A. (2017) 'Diagnosis and management of atherosclerotic cardiovascular disease in chronic kidney disease: a review.' *Kidney Int*, 91(4), Apr, 2017/01/04, pp. 797-807.

Mayer, B. and Mayer, B. (2014) 'How much nicotine kills a human? Tracing back the generally accepted lethal dose to dubious self-experiments in the nineteenth century.' *Archives of Toxicology*, 88(1) pp. 5-7.

McFadyen, Kiefer, J., Braig, D., Loseff-Silver, J., Potempa, L. A., Eisenhardt, S. U. and Peter, K. (2018) 'Dissociation of C-Reactive Protein Localizes and Amplifies Inflammation: Evidence for a Direct Biological Role of C-Reactive Protein and Its Conformational Changes.' *Frontiers in immunology*, 9 p. 1351.

McLaughlin, M. M., Kumar, S., McDonnell, P. C., Van Horn, S., Lee, J. C., Livi, G. P. and Young, P. R. (1996) 'Identification of mitogen-activated protein (MAP) kinase-activated

protein kinase-3, a novel substrate of CSBP p38 MAP kinase.' *J Biol Chem*, 271(14), Apr 5, 1996/04/05, pp. 8488-8492.

Meier, R. P. H., Meyer, J., Montanari, E., Lacotte, S., Balaphas, A., Muller, Y. D., Clement, S., Negro, F., et al. (2019) 'Interleukin-1 Receptor Antagonist Modulates Liver Inflammation and Fibrosis in Mice in a Model-Dependent Manner.' *Int J Mol Sci*, 20(6), Mar 14, 2019/03/17,

Meijer, C., Huysen, V., Smeenk, R. T. and Swaak, A. J. (1993) 'Profiles of cytokines (TNF alpha and IL-6) and acute phase proteins (CRP and alpha 1AG) related to the disease course in patients with systemic lupus erythematosus.' *Lupus*, 2(6), Dec, 1993/12/01, pp. 359-365.

Meschia, J. F. and Brott, T. (2018) 'Ischaemic stroke.' *European Journal of Neurology*, 25(1) pp. 35-40.

Metz and Tracey, K. J. (2005) 'It takes nerve to dampen inflammation.' *Nature Immunology*, 6(8) pp. 756-757.

Mihara, T., Otsubo, W., Horiguchi, K., Mikawa, S., Kaji, N., Iino, S., Ozaki, H. and Hori, M. (2017) 'The anti-inflammatory pathway regulated via nicotinic acetylcholine receptors in rat intestinal mesothelial cells.' *J Vet Med Sci*, 79(11), Nov 10, 2017/09/22, pp. 1795-1802.

Mijajlović, M. D., Pavlović, A., Brainin, M., Heiss, W.-D., Quinn, T. J., Ihle-Hansen, H. B., Hermann, D. M., Assayag, E. B., et al. (2017) 'Post-stroke dementia - a comprehensive review.' *BMC Medicine*, 15(1)

Mijatovic, T., Houzet, L., Defrance, P., Droogmans, L., Huez, G. and Kruys, V. (2000) 'Tumor necrosis factor-alpha mRNA remains unstable and hypoadenylated upon stimulation of macrophages by lipopolysaccharides.' *Eur J Biochem*, 267(19), Oct, 2000/09/21, pp. 6004-6012.

Milam. (2016) 'Exercise Guidelines for Chronic Kidney Disease Patients.' *J Ren Nutr*, 26(4), Jul, 2016/06/19, pp. e23-25.

Mills. (2001) 'Macrophage arginine metabolism to ornithine/urea or nitric oxide/citrulline: a life or death issue.' *Crit Rev Immunol*, 21(5) 2002/04/11, pp. 399-425.

Mills, Kincaid, K., Alt, J. M., Heilman, M. J. and Hill, A. M. (2000) 'M-1/M-2 Macrophages and the Th1/Th2 Paradigm.' *The Journal of Immunology*, 164(12) pp. 6166-6173.

Mirza, R. and Koh, T. J. (2011) 'Dysregulation of monocyte/macrophage phenotype in wounds of diabetic mice.' *Cytokine*, 56(2) pp. 256-264.

Mirzaei, A. and Mahmoudi, H. (2018) 'Evaluation of TNF-alpha cytokine production in patients with tuberculosis compared to healthy people.' *GMS Hyg Infect Control*, 13 2018/12/28, p. Doc09.

Mkaddem, Benhamou, M. and Monteiro, R. C. (2019) 'Understanding Fc Receptor Involvement in Inflammatory Diseases: From Mechanisms to New Therapeutic Tools.' *Front Immunol*, 10 2019/05/07, p. 811.

Moens, U., Kostenko, S. and Sveinbjornsson, B. (2013) 'The Role of Mitogen-Activated Protein Kinase-Activated Protein Kinases (MAPKAPKs) in Inflammation.' *Genes (Basel)*, 4(2), Mar 26, 2013/01/01, pp. 101-133.

Mold, C., Nakayama, S., Holzer, T. J., Gewurz, H. and Du Clos, T. W. (1981) 'C-reactive protein is protective against Streptococcus pneumoniae infection in mice.' *The Journal of experimental medicine*, 154(5) p. 1703.

Molins, Peña, E., Vilahur, G., Mendieta, C., Slevin, M. and Badimon, L. (2008) 'C-Reactive Protein Isoforms Differ in Their Effects on Thrombus Growth.' *Arteriosclerosis, thrombosis, and vascular biology*, 28(12) pp. 2239-2246.

Molins, Anna Pascual, M., Llorenc, V., Zarranz-Ventura, J., Mesquida, M., Adan, A. and Martorell, J. (2017) 'C-reactive protein isoforms differentially affect outer blood-retinal barrier integrity and function.' *AMERICAN JOURNAL OF PHYSIOLOGY-CELL PHYSIOLOGY*, 312(3) pp. C244-C253.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L. and O'Garra, A. (2001) 'INTERLEUKIN-10 AND THE INTERLEUKIN-10 RECEPTOR.' *Annual Review of Immunology*, 19(1) pp. 683-765.

Morris, Goodsell, D. S., Halliday, R. S., Huey, R., Hart, W. E., Belew, R. K. and Olson, A. J. (1998) 'Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function.' *Journal of Computational Chemistry*, 19(14) pp. 1639-1662.

Morris, Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S. and Olson, A. J. (2009) 'AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility.' *J Comput Chem*, 30(16), Dec, 2009/04/29, pp. 2785-2791.

Morrison, D. K. (2012) 'MAP kinase pathways.' *Cold Spring Harb Perspect Biol*, 4(11), Nov 1, 2012/11/06,

Mortensen. (2001) 'C-reactive protein, inflammation, and innate immunity.' *Immunol Res*, 24(2) 2001/10/12, pp. 163-176.

Moshage, H. (1997) 'Cytokines and the hepatic acute phase response.' *J Pathol*, 181(3), Mar, 1997/03/01, pp. 257-266.

Motwani, M. P. and Gilroy, D. W. (2015) 'Macrophage development and polarization in chronic inflammation.' *Seminars in Immunology*, 27(4) pp. 257-266.

Mugabo, Y., Li, L. and Renier, G. (2010) 'The connection between C-reactive protein (CRP) and diabetic vasculopathy. Focus on preclinical findings.' *Current diabetes reviews U6 - US U7 - Journal Article*, 6(1) p. 27.

Muhl, H. and Pfeilschifter, J. (2003) 'Anti-inflammatory properties of pro-inflammatory interferon-gamma.' *Int Immunopharmacol*, 3(9), Sep, 2003/08/02, pp. 1247-1255.

Murphy, M. P. and LeVine, H., 3rd. (2010) 'Alzheimer's disease and the amyloid-beta peptide.' *J Alzheimers Dis*, 19(1) 2010/01/12, pp. 311-323.

Murray and Wynn, T. A. (2011) 'Protective and pathogenic functions of macrophage subsets.' *Nature Reviews Immunology*, 11(11) pp. 723-737.

Murray and Reardon, C. (2018) 'The cholinergic anti-inflammatory pathway revisited.' *Neurogastroenterol Motil*, 30(3), Mar, 2018/02/23,

Nadeau, V., Guillemette, S., Belanger, L. F., Jacob, O., Roy, S. and Charron, J. (2009) 'Map2k1 and Map2k2 genes contribute to the normal development of syncytiotrophoblasts during placentation.' *Development*, 136(8), Apr, 2009/03/24, pp. 1363-1374.

Nahrendorf, M., Pittet, M. J. and Swirski, F. K. (2010) 'Monocytes: protagonists of infarct inflammation and repair after myocardial infarction.' *Circulation*, 121(22), Jun 8, 2010/06/10, pp. 2437-2445.

Naicker, S. D., Cormican, S., Griffin, T. P., Maretto, S., Martin, W. P., Ferguson, J. P., Cotter, D., Connaughton, E. P., et al. (2018) 'Chronic Kidney Disease Severity Is Associated With Selective Expansion of a Distinctive Intermediate Monocyte Subpopulation.' *Front Immunol*, 9 2019/01/09, p. 2845.

Napoli, D., Slevin, M., Popa-Wagner, A., Singh, P., Lattanzi, S. and Divani, A. A. (2018) 'Monomeric C-Reactive Protein and Cerebral Hemorrhage: From Bench to Bedside.' *Frontiers in immunology*, 9 p. 1921.

Nathan. (2002) 'Points of control in inflammation.' *Nature*, 420(6917) pp. 846-852.

Nazarov, P. G., Krylova, I. B., Evdokimova, N. R., Nezhinskaya, G. I. and Butyugov, A. A. (2007) 'C-reactive protein: A pentraxin with anti-acetylcholine activity.' *Life Sciences*, 80(24-25) pp. 2337-2341.

Neamatallah. (2019) 'Mitogen-Activated Protein Kinase Pathway: A Critical Regulator in Tumor-associated Macrophage Polarization.' *J Microsc Ultrastruct*, 7(2), Apr-Jun, 2019/07/12, pp. 53-56.

Ngkelo, A., Meja, K., Yeadon, M., Adcock, I. and Kirkham, P. A. (2012) 'LPS induced inflammatory responses in human peripheral blood mononuclear cells is mediated through NOX4 and Giα dependent PI-3kinase signalling.' *J Inflamm (Lond)*, 9(1), Jan 12, 2012/01/14, p. 1.

Niikura, T., Tajima, H. and Kita, Y. (2006) 'Neuronal cell death in Alzheimer's disease and a neuroprotective factor, humanin.' *Curr Neuropharmacol*, 4(2), Apr, 2008/07/11, pp. 139-147.

Nording, H. M., Seizer, P. and Langer, H. F. (2015) 'Platelets in inflammation and atherogenesis.' *Front Immunol*, 6 2015/03/24, p. 98.

North, Venter, C. S. and Jerling, J. C. (2009) 'The effects of dietary fibre on C-reactive protein, an inflammation marker predicting cardiovascular disease.' *Eur J Clin Nutr*, 63(8), Aug, 2009/02/19, pp. 921-933.

O'Brien, J. T. and Thomas, A. (2015) 'Vascular dementia.' *LANCET*, 386(10004) pp. 1698-1706.

O'Rourke, S. A., Dunne, A. and Monaghan, M. G. (2019) 'The Role of Macrophages in the Infarcted Myocardium: Orchestrators of ECM Remodeling.' *Front Cardiovasc Med*, 6 2019/08/17, p. 101.

Obata, T., Brown, G. E. and Yaffe, M. B. (2000) 'MAP kinase pathways activated by stress: the p38 MAPK pathway.' *Crit Care Med*, 28(4 Suppl), Apr, 2000/05/12, pp. N67-77.

Oeckinghaus and Ghosh, S. (2009) 'The NF-kappaB family of transcription factors and its regulation.' *Cold Spring Harb Perspect Biol*, 1(4), Oct, 2010/01/13, p. a000034.

Ogle, M. E., Segar, C. E., Sridhar, S. and Botchwey, E. A. (2016) 'Monocytes and macrophages in tissue repair: Implications for immunoregenerative biomaterial design.' *Experimental Biology and Medicine*, 241(10) pp. 1084-1097.

Ohara, Y., Yabuki, A., Nakamura, R., Ichii, O., Mizukawa, H., Yokoyama, N. and Yamato, O. (2019) 'Renal Infiltration of Macrophages in Canine and Feline Chronic Kidney Disease.' *J Comp Pathol*, 170, Jul, 2019/08/04, pp. 53-59.

Oke, S. L. and Tracey, K. J. (2008) 'From CNI-1493 to the immunological homunculus: physiology of the inflammatory reflex.' *Journal of Leukocyte Biology*, 83(3) pp. 512-517.

Okun, E., Mattson, M. P. and Arumugam, T. V. (2010) 'Involvement of Fc receptors in disorders of the central nervous system.' *Neuromolecular Med*, 12(2), Jun, 2009/10/22, pp. 164-178.

Olechnowicz-Tietz, S., Gluba, A., Paradowska, A., Banach, M. and Rysz, J. (2013) 'The risk of atherosclerosis in patients with chronic kidney disease.' *Int Urol Nephrol*, 45(6), Dec, 2013/03/14, pp. 1605-1612.

Orekhov, A. N., Orekhova, V. A., Nikiforov, N. G., Myasoedova, V. A., Grechko, A. V., Romanenko, E. B., Zhang, D. and Chistiakov, D. A. (2019) 'Monocyte differentiation and macrophage polarization.' *Vessel Plus*, 3 p. 10.

Orr-Urtreger, A., Göldner, F. M., Saeki, M., Lorenzo, I., Goldberg, L., De Biasi, M., Dani, J. A., Patrick, J. W., et al. (1997) 'Mice Deficient in the α7 Neuronal Nicotinic Acetylcholine Receptor Lack α-Bungarotoxin Binding Sites and Hippocampal Fast Nicotinic Currents.' *The Journal of Neuroscience*, 17(23) pp. 9165-9171.

Osmand, A. P., Friedenson, B., Gewurz, H., Painter, R. H., Hofmann, T. and Shelton, E. (1977) 'Characterization of C-Reactive Protein and the Complement Subcomponent C1t as Homologous Proteins Displaying Cyclic Pentameric Symmetry (Pentraxins).' *Proceedings of the National Academy of Sciences of the United States of America*, 74(2) pp. 739-743.

Ozanska, A., Szymczak, D. and Rybka, J. (2020) 'Pattern of human monocyte subpopulations in health and disease.' *Scand J Immunol*, 92(1), Jul, 2020/04/04, p. e12883.

P, H., E, M., G, F. and D, M. (2003) 'Chemokines, chemokine receptors and adhesion molecules on different human endothelia: discriminating the tissue-specific functions that affect leucocyte migration.' *Clinical & Experimental Immunology*, 134(3) pp. 431-441.

Paepegaey, A.-C., Genser, L., Bouillot, J.-L., Oppert, J.-M., Clément, K. and Poitou, C. (2015) 'High levels of CRP in morbid obesity: the central role of adipose tissue and lessons for

clinical practice before and after bariatric surgery.' *Surgery for obesity and related diseases U6 US U7 - Journal Article*, 11(1) p. 148.

Paffen and DeMaat, M. P. (2006) 'C-reactive protein in atherosclerosis: A causal factor?' *Cardiovasc Res*, 71(1), Jul 1, 2006/04/21, pp. 30-39.

Paffen, Vos, H. L. and Bertina, R. M. (2004) 'C-Reactive Protein Does Not Directly Induce Tissue Factor in Human Monocytes.' *Arteriosclerosis, Thrombosis, and Vascular Biology: Journal of the American Heart Association*, 24(5) pp. 975-981.

Palou, A. and Bonet, M. L. (2013) 'Challenges in obesity research.' *Nutr Hosp*, 28 Suppl 5, Sep, 2013/10/22, pp. 144-153.

Panee, J. (2012) 'Monocyte Chemoattractant Protein 1 (MCP-1) in obesity and diabetes.' *Cytokine*, 60(1), Oct, 2012/07/07, pp. 1-12.

Pant, S., Deshmukh, A., GuruMurthy, G. S., Pothineni, N. V., Watts, T. E., Romeo, F. and Mehta, J. L. (2014) 'Inflammation and Atherosclerosis—Revisited.' *Journal of Cardiovascular Pharmacology and Therapeutics*, 19(2) pp. 170-178.

Papa, S., Choy, P. M. and Bubici, C. (2019) 'The ERK and JNK pathways in the regulation of metabolic reprogramming.' *Oncogene*, 38(13), Mar, 2018/11/30, pp. 2223-2240.

Parameswaran, N. and Patial, S. (2010) 'Tumor necrosis factor-alpha signaling in macrophages.' *Crit Rev Eukaryot Gene Expr*, 20(2) 2010/12/08, pp. 87-103.

Park, Aziz, N. and Cho, J. Y. (2019) 'MKK7, the essential regulator of JNK signaling involved in cancer cell survival: a newly emerging anticancer therapeutic target.' *Ther Adv Med Oncol*, 11 2019/10/04, p. 1758835919875574.

Park, Lee, P. H., Ahn, Y. W., Choi, Y. J., Lee, G., Lee, D.-Y., Chung, E. S. and Jin, B. K. (2007) 'Neuroprotective effect of nicotine on dopaminergic neurons by anti-inflammatory action.' *European Journal of Neuroscience*, 26(1) pp. 79-89.

Parnell, E., Smith, B. O., Palmer, T. M., Terrin, A., Zaccolo, M. and Yarwood, S. J. (2012) 'Regulation of the inflammatory response of vascular endothelial cells by EPAC1.' *Br J Pharmacol*, 166(2), May, 2011/12/08, pp. 434-446.

Pasceri, V., Willerson, J. T. and Yeh, E. T. (2000a) 'Direct proinflammatory effect of C-reactive protein on human endothelial cells.' *Circulation*, 102(18), Oct 31, 2000/11/01, pp. 2165-2168.

Pasceri, V., Wu, H. D., Willerson, J. T. and Yeh, E. T. (2000b) 'Modulation of vascular inflammation in vitro and in vivo by peroxisome proliferator-activated receptor-gamma activators.' *Circulation*, 101(3), Jan 25, 2000/01/25, pp. 235-238.

Pasceri, V., Cheng, J. S., Willerson, J. T. and Yeh, E. T. (2001) 'Modulation of C-reactive protein-mediated monocyte chemoattractant protein-1 induction in human endothelial cells by anti-atherosclerosis drugs.' *Circulation*, 103(21), May 29, 2001/05/31, pp. 2531-2534.

Pase, M. P., Himali, J. J., Beiser, A. S., Aparicio, H. J., Satizabal, C. L., Vasan, R. S., Seshadri, S. and Jacques, P. F. (2017) 'Sugar- and Artificially Sweetened Beverages and the Risks of Incident Stroke and Dementia: A Prospective Cohort Study.' *Stroke*, 48(5) pp. 1139-1146.

Passmore, J. S., Lukey, P. T. and Ress, S. R. (2001) 'The human macrophage cell line U937 as an in vitro model for selective evaluation of mycobacterial antigen-specific cytotoxic T-cell function.' *Immunology*, 102(2) pp. 146-156.

Pastorino, U., Morelli, D., Leuzzi, G., Gisabella, M., Suatoni, P., Taverna, F., Bertocchi, E., Boeri, M., et al. (2017) 'Baseline and postoperative C-reactive protein levels predict mortality in operable lung cancer.' *European Journal of Cancer*, 79 pp. 90-97.

Pate, M., Damarla, V., Chi, D. S., Negi, S. and Krishnaswamy, G. (2010) 'Endothelial cell biology: role in the inflammatory response.' *Advances in clinical chemistry*, 52 p. 109.

Patel, H., McIntire, J., Ryan, S., Dunah, A. and Loring, R. (2017) 'Anti-inflammatory effects of astroglial α7 nicotinic acetylcholine receptors are mediated by inhibition of the NF-κB pathway and activation of the Nrf2 pathway.' *Journal of neuroinflammation*, 14(1) pp. 192-115.

Paulsen, O., Laird, B., Aass, N., Lea, T., Fayers, P., Kaasa, S. and Klepstad, P. (2017) 'The relationship between pro-inflammatory cytokines and pain, appetite and fatigue in patients with advanced cancer.' *PLOS ONE*, 12(5) p. e0177620.

Pavlov and Tracey, K. J. (2005) 'The cholinergic anti-inflammatory pathway.' *Brain Behavior and Immunity*, 19(6) pp. 493-499.

Pavlov and Tracey, K. J. (2012) 'The vagus nerve and the inflammatory reflex - Linking immunity and metabolism.' *Nature Reviews Endocrinology*, 8(12) pp. 743-754.

Pecht, T., Gutman-Tirosh, A., Bashan, N. and Rudich, A. (2014) 'Peripheral blood leucocyte subclasses as potential biomarkers of adipose tissue inflammation and obesity subphenotypes in humans.' *Obes Rev*, 15(4), Apr, 2013/11/21, pp. 322-337.

Pedragosa, J., Salas-Perdomo, A., Gallizioli, M., Cugota, R., Miro-Mur, F., Brianso, F., Justicia, C., Perez-Asensio, F., et al. (2018) 'CNS-border associated macrophages respond to acute ischemic stroke attracting granulocytes and promoting vascular leakage.' *ACTA NEUROPATHOLOGICA COMMUNICATIONS*, 6(1) pp. 1-19.

Peña, E., la Torre, R. d., Arderiu, G., Slevin, M. and Badimon, L. (2017) 'mCRP triggers angiogenesis by inducing F3 transcription and TF signalling in microvascular endothelial cells.' *Thrombosis and Haemostasis*, 117(2) pp. 357-370.

Pepys and Hirschfield. (2003) 'C-reactive protein: a critical update.' *J Clin Invest*, 111(12), Jun, 2003/06/19, pp. 1805-1812.

Pepys, Hawkins, P. N., Kahan, M. C., Tennent, G. A., Gallimore, J. R., Graham, D., Sabin, C. A., Zychlinsky, A., et al. (2005) 'Proinflammatory Effects of Bacterial Recombinant Human C-Reactive Protein Are Caused by Contamination With Bacterial Products, Not by C-Reactive Protein Itself.' *Circulation research*, 97(11) pp. e97-e103.

Pepys, Hirschfield, Glenys A. Tennent, J. Ruth Gallimore, Melvyn C. Kahan, Vittorio Bellotti, Philip N. Hawkins, Rebecca M. Myers, et al. (2006) 'Targeting C-reactive protein for the treatment of cardiovascular disease.' *Nature*, 440(7088) pp. 1217-1221.

Pilling, D., Galvis-Carvajal, E., Karhadkar, T. R., Cox, N. and Gomer, R. H. (2017) 'Monocyte differentiation and macrophage priming are regulated differentially by pentraxins and their ligands.' *BMC Immunol*, 18(1), Jun 15, 2017/06/18, p. 30.

Pires, Silva, R., Ferreira, G. M. and Abdelhay, E. (2018) 'NF-kappaB: Two Sides of the Same Coin.' *GENES*, 9(1) p. 24.

Pisters, R. and Lip, G. Y. (2013) 'Safety and efficacy of new anticoagulants in patients with heart failure.' *Curr Heart Fail Rep*, 10(1), Mar, 2012/12/12, pp. 18-25.

Plotnikov, A., Zehorai, E., Procaccia, S. and Seger, R. (2011) 'The MAPK cascades: Signaling components, nuclear roles and mechanisms of nuclear translocation.' *Biochimica et Biophysica Acta - Molecular Cell Research*, 1813(9) pp. 1619-1633.

Poirier, P., Giles, T. D., Bray, G. A., Hong, Y., Stern, J. S., Pi-Sunyer, F. X. and Eckel, R. H. (2006) 'Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss.' *Arterioscler Thromb Vasc Biol*, 26(5), May, 2006/04/22, pp. 968-976.

Poitou, C., Dalmas, E., Renovato, M., Benhamo, V., Hajduch, F., Abdennour, M., Kahn, J.-F., Veyrie, N., et al. (2011) 'CD14dimCD16+ and CD14+CD16+ Monocytes in Obesity and During Weight Loss: Relationships With Fat Mass and Subclinical Atherosclerosis.' *Arteriosclerosis, thrombosis, and vascular biology*, 31(10) pp. 2322-2330.

Potempa, L. A., Maldonado, B. A., Laurent, P., Zemel, E. S. and Gewurz, H. (1983) 'Antigenic, electrophoretic and binding alterations of human C-reactive protein modified selectively in the absence of calcium.' *Molecular Immunology*, 20(11) pp. 1165-1175.

Potempa, L. A., Yao, Z. Y., Ji, S. R., Filep, J. G. and Wu, Y. (2015) 'Solubilization and purification of recombinant modified C-reactive protein from inclusion bodies using reversible anhydride modification.' *Biophys Rep*, 1 pp. 18-33.

Poulsen, M. M., Fjeldborg, K., Ornstrup, M. J., Kjær, T. N., Nøhr, M. K. and Pedersen, S. B. (2015) 'Resveratrol and inflammation: Challenges in translating pre-clinical findings to improved patient outcomes.' *BBA - Molecular Basis of Disease*, 1852(6) pp. 1124-1136.

Póvoa, P., Coelho, L., Almeida, E., Fernandes, A., Mealha, R., Moreira, P. and Sabino, H. (2005) 'C-reactive protein as a marker of infection in critically ill patients.' *Clinical Microbiology and Infection*, 11(2) pp. 101-108.

Power, C., Henry, S., Del Bigio, M. R., Larsen, P. H., Corbett, D., Imai, Y., Yong, V. W. and Peeling, J. (2003) 'Intracerebral hemorrhage induces macrophage activation and matrix metalloproteinases.' *Annals of neurology*, 53(6) pp. 731-742.

Prince, L. R., Whyte, M. K., Sabroe, I. and Parker, L. C. (2011) 'The role of TLRs in neutrophil activation.' *Curr Opin Pharmacol*, 11(4), Aug, 2011/07/12, pp. 397-403.

Profita, M., Bonanno, A., Siena, L., Ferraro, M., Montalbano, A. M., Pompeo, F., Riccobono, L., Pieper, M. P., et al. (2008) 'Acetylcholine mediates the release of IL-8 in human bronchial

epithelial cells by a NFkB/ERK-dependent mechanism.' *Eur J Pharmacol*, 582(1-3), Mar 17, 2008/02/05, pp. 145-153.

Qin, Zhou, L.-Q., Ma, X.-T., Hu, Z.-W., Yang, S., Chen, M., Bosco, D. B., Wu, L.-J., et al. (2019) 'Dual Functions of Microglia in Ischemic Stroke.' *Neuroscience bulletin*,

Quik, M., Zhang, D., McGregor, M. and Bordia, T. (2015) 'Alpha7 nicotinic receptors as therapeutic targets for Parkinson's disease.' *Biochem Pharmacol*, 97(4), Oct 15, 2015/06/21, pp. 399-407.

Rader, D. J. (2000) 'Inflammatory markers of coronary risk.' *N Engl J Med*, 343(16), Oct 19, 2000/10/19, pp. 1179-1182.

Ramadan, A., Land, W. G. and Paczesny, S. (2017) 'Editorial: Danger Signals Triggering Immune Response and Inflammation.' *Front Immunol*, 8 2017/08/30, p. 979.

Ramana, K. V. (2010) 'Tumor necrosis factor-alpha converting enzyme: Implications for ocular inflammatory diseases.' *Int J Biochem Cell Biol*, 42(7), Jul, 2010/03/23, pp. 1076-1079.

Rampersad, S. N. (2012) 'Multiple applications of alamar blue as an indicator of metabolic function and cellular health in cell viability bioassays.' *Sensors (Switzerland)*, 12(9) pp. 12347-12360.

Ratajczak-Wrona, W., Jablonska, E., Marcinczyk, M., Grabowska, Z. and Piotrowski, L. (2009) 'Role of p38 MAPK pathway in induction of iNOS expression in neutrophils and peripheral blood mononuclear cells in patients with squamous cell carcinoma of the oral cavity.' *J Oral Maxillofac Surg*, 67(11), Nov, 2009/10/20, pp. 2354-2363.

Reiss, Siegart, N. M. and De Leon, J. (2017) 'Interleukin-6 in atherosclerosis: atherogenic or atheroprotective?' *Clinical lipidology*, 12(1) pp. 14-23.

Ren, C., Tong, Y. L., Li, J. C., Lu, Z. Q. and Yao, Y. M. (2017) 'The Protective Effect of Alpha 7 Nicotinic Acetylcholine Receptor Activation on Critical Illness and Its Mechanism.' *Int J Biol Sci*, 13(1) 2017/01/27, pp. 46-56.

Ridker, P. M., Hennekens, C. H., Buring, J. E. and Rifai, N. (2000) 'C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women.' *New England Journal of Medicine*, 342(12) pp. 836-843.

Robak, T., Gladalska, A. and Stepień, H. (1998) 'The tumour necrosis factor family of receptors/ligands in the serum of patients with rheumatoid arthritis.' *Eur Cytokine Netw*, 9(2), Jun, 1998/07/29, pp. 145-154.

Rodero and Khosrotehrani, K. (2010) 'Skin wound healing modulation by macrophages.' *Int J Clin Exp Pathol*, 3(7), Jul 25, 2010/09/11, pp. 643-653.

Rodero, Licata, F., Poupel, L., Hamon, P., Khosrotehrani, K., Combadiere, C. and Boissonnas, A. (2014) 'In vivo imaging reveals a pioneer wave of monocyte recruitment into mouse skin wounds.' *PLoS ONE*, 9(10) p. e108212.

Rodriguez-Hernandez, H., Simental-Mendia, L. E., Rodriguez-Ramirez, G. and Reyes-Romero, M. A. (2013) 'Obesity and inflammation: epidemiology, risk factors, and markers of inflammation.' *Int J Endocrinol*, 2013 2013/05/22, p. 678159.

Rogacev, Zawada, A. M., Emrich, I., Seiler, S., Böhm, M., Fliser, D., Woollard, K. J. and Heine, G. H. (2014) 'Lower Apo A-I and Lower HDL-C Levels Are Associated With Higher Intermediate CD14++CD16+ Monocyte Counts That Predict Cardiovascular Events in Chronic Kidney Disease.' *Arteriosclerosis, thrombosis, and vascular biology*, 34(9) pp. 2120-2127.

Rogacev, Ulrich, C., Blomer, L., Hornof, F., Oster, K., Ziegelin, M., Cremers, B., Grenner, Y., et al. (2010) 'Monocyte heterogeneity in obesity and subclinical atherosclerosis.' *Eur Heart J*, 31(3), Feb, 2009/08/19, pp. 369-376.

Roger, V. L., Go, A. S., Lloyd-Jones, D. M., Adams, R. J., Berry, J. D., Brown, T. M., Carnethon, M. R., Dai, S., et al. (2011) 'Heart disease and stroke statistics-2011 update: A report from the American Heart Association.' *Circulation*, 123(4) pp. e18-e19.

Roh, J. S. and Sohn, D. H. (2018) 'Damage-Associated Molecular Patterns in Inflammatory Diseases.' *Immune Netw*, 18(4), Aug, 2018/09/06, p. e27.

Rosas-Ballina and Tracey, K. J. (2009) 'Cholinergic control of inflammation.' *J Intern Med*, 265(6), Jun, 2009/06/06, pp. 663-679.

Rosas-Ballina, Olofsson, P. S., Ochani, M., Valdés-Ferrer, S. I., Levine, Y. A., Reardon, C., Tusche, M. W., Pavlov, V. A., et al. (2011) 'Acetylcholine-Synthesizing T Cells Relay Neural Signals in a Vagus Nerve Circuit.' *Science*, 334(6052) pp. 98-101.

Rost, N. S., Wolf, P. A., Kase, C. S., Kelly-Hayes, M., Silbershatz, H., Massaro, J. M., D'Agostino, R. B., Franzblau, C., et al. (2001) 'Plasma concentration of C-reactive protein and risk of ischemic stroke and transient ischemic attack: The Framingham Study.' *Stroke*, 32(11) pp. 2575-2579.

Rothe, G., Gabriel, H., Kovacs, E., Klucken, J., Stohr, J., Kindermann, W. and Schmitz, G. (1996) 'Peripheral blood mononuclear phagocyte subpopulations as cellular markers in hypercholesterolemia.' *Arterioscler Thromb Vasc Biol*, 16(12), Dec, 1996/12/01, pp. 1437-1447.

Sacco, R. L., Kasner, S. E., Broderick, J. P., Caplan, L. R., Connors, J. J., Culebras, A., Elkind, M. S. V., George, M. G., et al. (2013) 'An Updated Definition of Stroke for the 21st Century: A Statement for Healthcare Professionals From the American Heart Association/American Stroke Association.' *Stroke*, 44(7) pp. 2064-2089.

Saeed, R. W., Varma, S., Peng-Nemeroff, T., Sherry, B., Balakhaneh, D., Huston, J., Tracey, K. J., Al-Abed, Y., et al. (2005) 'Cholinergic stimulation blocks endothelial cell activation and leukocyte recruitment during inflammation.' *The Journal of experimental medicine*, 201(7) p. 1113.

Sahbudak Bal, Z., Karadas Ozdemir, N., Sen, S., Yilmaz Karapinar, D., Azarsiz, E., Aydemir, S. and Vardar, F. (2017) 'Diagnostic Accuracy of Interleukin-6, Interleukin-8, and Interleukin-10 for Predicting Bacteremia in Children with Febrile Neutropenia.' *Turk J Haematol*, 34(3), Aug 2, 2017/02/06, pp. 254-257.

Sakurai, H. (2012) 'Targeting of TAK1 in inflammatory disorders and cancer.' *Trends Pharmacol Sci*, 33(10), Oct, 2012/07/17, pp. 522-530.

Salazar, J., Martínez, M. S., Chávez-Castillo, M., Núñez, V., Añez, R., Torres, Y., Toledo, A., Chacín, M., et al. (2014) 'C-Reactive Protein: An In-Depth Look into Structure, Function, and Regulation.' *International Scholarly Research Notices*, 2014 pp. 1-11.

Sampath, P., Moideen, K., Ranganathan, U. D. and Bethunaickan, R. (2018) 'Monocyte Subsets: Phenotypes and Function in Tuberculosis Infection.' *Front Immunol*, 9 2018/08/15, p. 1726.

Sargent, P. B. (1993) 'The diversity of neuronal nicotinic acetylcholine receptors.' *Annu Rev Neurosci*, 16 1993/01/01, pp. 403-443.

Satpathy, A. T., Wu, X., Albring, J. C. and Murphy, K. M. (2012) 'Re(de)fining the dendritic cell lineage.' *Nature Immunology*, 13(12) pp. 1145-1154.

Sattar, N., Perera, M., Small, M. and Lumsden, M. A. (1999) 'Hormone replacement therapy and sensitive C-reactive protein concentrations in women with type-2 diabetes.' *Lancet*, 354(9177), Aug 7, 1999/08/28, pp. 487-488.

Schipper, H. S., Nuboer, R., Prop, S., van den Ham, H. J., de Boer, F. K., Kesmir, C., Mombers, I. M. H., van Bekkum, K. A., et al. (2012) 'Systemic inflammation in childhood obesity: circulating inflammatory mediators and activated CD14++ monocytes.' *Diabetologia*, 55(10), Oct, 2012/07/19, pp. 2800-2810.

Schmidt-Arras, D. and Rose-John, S. (2016) 'IL-6 pathway in the liver: From physiopathology to therapy.' *J Hepatol*, 64(6), Jun, 2016/02/13, pp. 1403-1415.

Schoenborn, J. R. and Wilson, C. B. (2007) *Regulation of Interferon-γ During Innate and Adaptive Immune Responses.* Vol. 96. SAN DIEGO, 2007. ELSEVIER ACADEMIC PRESS INC.

Schonbeck, U. and Libby, P. (2001) 'The CD40/CD154 receptor/ligand dyad.' *Cell Mol Life Sci*, 58(1), Jan, 2001/03/07, pp. 4-43.

Schwedler, Guderian, F., Dammrich, J., Potempa, L. A. and Wanner, C. (2003) 'Tubular staining of modified C-reactive protein in diabetic chronic kidney disease.' *Nephrol Dial Transplant*, 18(11), Nov, 2003/10/11, pp. 2300-2307.

Scull, C. M., Hays, W. D. and Fischer, T. H. (2010) 'Macrophage pro-inflammatory cytokine secretion is enhanced following interaction with autologous platelets.' *Journal of inflammation (London, England)*, 7(1) pp. 53-53.

Seger, R. and Krebs, E. G. (1995) 'The MAPK signaling cascade.' *FASEB J*, 9(9), Jun, 1995/06/01, pp. 726-735.

Seger, R., Biener, Y., Feinstein, R., Hanoch, T., Gazit, A. and Zick, Y. (1995) 'Differential activation of mitogen-activated protein kinase and S6 kinase signaling pathways by 12-O-tetradecanoylphorbol-13-acetate (TPA) and insulin. Evidence for involvement of a TPA-stimulated protein-tyrosine kinase.' *J Biol Chem*, 270(47), Nov 24, 1995/11/24, pp. 28325-28330.

Seger, R., Ahn, N. G., Posada, J., Munar, E. S., Jensen, A. M., Cooper, J. A., Cobb, M. H. and Krebs, E. G. (1992) 'Purification and characterization of mitogen-activated protein kinase activator(s) from epidermal growth factor-stimulated A431 cells.' *Journal of Biological Chemistry*, 267(20) p. 14373.

Seki, E., Brenner, D. A. and Karin, M. (2012) 'A liver full of JNK: signaling in regulation of cell function and disease pathogenesis, and clinical approaches.' *Gastroenterology*, 143(2), Aug, 2012/06/19, pp. 307-320.

Serra, M. B., Barroso, W. A., da Silva, N. N., Silva, S. D. N., Borges, A. C. R., Abreu, I. C. and Borges, M. (2017) 'From Inflammation to Current and Alternative Therapies Involved in Wound Healing.' *Int J Inflam*, 2017 2017/08/16, p. 3406215.

Shao, Wu, H. J., Chen, S., Ammar, A. b., Zhang, J. M. and Hong, Y. (2014a) 'Resveratrol Attenuates Early Brain Injury After Subarachnoid Hemorrhage Through Inhibition of NF-κB-Dependent Inflammatory/MMP-9 Pathway.' *CNS Neuroscience & Therapeutics*, 20(2) pp. 182-185.

Shao, Cheng, Z., Li, X., Chernaya, V., Wang, H. and Yang, X. F. (2014b) 'Immunosuppressive/anti-inflammatory cytokines directly and indirectly inhibit endothelial dysfunction--a novel mechanism for maintaining vascular function.' *J Hematol Oncol*, 7, Oct 31, 2014/11/13, p. 80.

Shi and Pamer, E. G. (2011) 'Monocyte recruitment during infection and inflammation.' *Nature Reviews Immunology*, 11(11) pp. 762-774.

Shi, Zhao, L., Xu, C., Zhang, L. and Zhao, H. (2019) 'High Molecular Weight Hyaluronan Suppresses Macrophage M1 Polarization and Enhances IL-10 Production in PM(2.5)-Induced Lung Inflammation.' *Molecules*, 24(9), May 7, 2019/05/10,

Shih, Wang, C. Y. and Yang, C. M. (2015) 'NF-kappaB Signaling Pathways in Neurological Inflammation: A Mini Review.' *FRONTIERS IN MOLECULAR NEUROSCIENCE*, 8 p. 77.

Shimura, Kitagawa, M., Yamada, T., Ebi, M., Mizoshita, T., Tanida, S., Kataoka, H., Kamiya, T., et al. (2012) 'C-reactive protein is a potential prognostic factor for metastatic gastric cancer.' *Anticancer research U6 - US U7 - Journal Article*, 32(2) p. 491.

Shin, J. A., Lee, H., Lim, Y.-K., Koh, Y., Choi, J. H. and Park, E.-M. (2010) 'Therapeutic effects of resveratrol during acute periods following experimental ischemic stroke.' *Journal of Neuroimmunology*, 227(1) pp. 93-100.

Shrive, A. K., Cheetham, G. M., Holden, D., Myles, D. A., Turnell, W. G., Volanakis, J. E., Pepys, M. B., Bloomer, A. C., et al. (1996) 'Three dimensional structure of human C-reactive protein.' *Nature structural biology*, 3(4) p. 346.

Shrotriya, Walsh, D., Nowacki, A. S., Lorton, C., Aktas, A., Hullihen, B., Benanni-Baiti, N., Hauser, K., et al. (2018) 'Serum C-reactive protein is an important and powerful prognostic biomarker in most adult solid tumors.' *PloS one*, 13(8) p. e0202555.

Shytle, Mori, T., Townsend, K., Vendrame, M., Sun, N., Zeng, J., Ehrhart, J., Silver, A. A., et al. (2004) 'Cholinergic modulation of microglial activation by alpha 7 nicotinic receptors.' *J Neurochem*, 89(2), Apr, 2004/04/02, pp. 337-343.

Siasos, G., Tsigkou, V., Kokkou, E., Oikonomou, E., Vavuranakis, M., Vlachopoulos, C., Verveniotis, A., Limperi, M., et al. (2014) 'Smoking and atherosclerosis: mechanisms of disease and new therapeutic approaches.' *Curr Med Chem*, 21(34) 2014/09/02, pp. 3936-3948.

Simons, N., Mitchell, P., Dowling, R., Gonzales, M. and Yan, B. (2015) 'Thrombus composition in acute ischemic stroke: a histopathological study of thrombus extracted by endovascular retrieval.' *J Neuroradiol*, 42(2), Apr, 2014/02/25, pp. 86-92.

Sindrilaru, A. and Scharffetter-Kochanek, K. (2013) 'Disclosure of the Culprits: Macrophages—Versatile Regulators of Wound Healing.' *Advances in wound Care*, 2 pp. 357-368.

Singh, Agrawal, M. and Dore, S. (2013) 'Neuroprotective properties and mechanisms of resveratrol in in vitro and in vivo experimental cerebral stroke models.' *ACS Chem Neurosci*, 4(8), Aug 21, 2013/06/14, pp. 1151-1162.

Singh, Liu, X. and Ahmad, N. (2015) 'Resveratrol, in its natural combination in whole grape, for health promotion and disease management.' *Annals of the New York Academy of Sciences*, 1348(1) pp. 150-160.

Singh, Devaraj, S., Dasu, M. R., Ciobanu, D., Reusch, J. and Jialal, I. (2006) 'C-reactive protein decreases interleukin-10 secretion in activated human monocyte-derived macrophages via inhibition of cyclic AMP production.' *Arterioscler Thromb Vasc Biol*, 26(11), Nov, 2006/08/19, pp. 2469-2475.

Slevin and Molins, B. (2018) 'Editorial: C-Reactive Protein in Age-Related Disorders.' *Frontiers in immunology*, 9 p. 2745.

Slevin, Liu, D., Ferris, G., Al-Hsinawi, M., Al-Baradie, R. and Krupinski, J. (2017) 'Expression of Monomeric C-Reactive Protein in Infarcted Brain Tissue from Patients with Alzheimer's Disease.' *Turk Patoloji Derg*, 33(1) 2017/01/04, pp. 25-29.

Slevin, Matou-Nasri, S., Turu, M., Luque, A., Rovira, N., Badimon, L., Boluda, S., Potempa, L., et al. (2010) 'Modified C-Reactive Protein Is Expressed by Stroke Neovessels and Is a Potent Activator of Angiogenesis In Vitro.' *Brain Pathology*, 20(1) pp. 151-165.

Slevin, Iemma, R. S., Zeinolabediny, Y., Liu, D., Ferris, G. R., Caprio, V., Phillips, N., Di Napoli, M., et al. (2018) 'Acetylcholine Inhibits Monomeric C-Reactive Protein Induced Inflammation, Endothelial Cell Adhesion, and Platelet Aggregation; A Potential Therapeutic?' *Frontiers in Immunology*, 9

Slevin, Matou, S., Zeinolabediny, Y., Corpas, R., Weston, R., Liu, D., Boras, E., Di Napoli, M., et al. (2015) 'Monomeric C-reactive protein-a key molecule driving development of Alzheimer's disease associated with brain ischaemia?' *SCIENTIFIC REPORTS*, 5 p. 13281.

Song, M.-G., Ryoo, I.-G., Choi, H.-Y., Choi, B.-H., Kim, S.-T., Heo, T.-H., Lee, J. Y., Park, P.-H., et al. (2015) 'NRF2 Signaling Negatively Regulates Phorbol-12-Myristate-13-Acetate (PMA)-

Induced Differentiation of Human Monocytic U937 Cells into Pro-Inflammatory Macrophages.' *PloS one*, 10(7) p. e0134235.

Sproston and Ashworth, J. J. (2018) 'Role of C-Reactive Protein at Sites of Inflammation and Infection.'

Sproston, El Mohtadi, M., Slevin, M., Gilmore, W. and Ashworth, J. J. (2018) 'The effect of C-reactive Protein Isoforms on nitric Oxide Production by U937 Monocytes/Macrophages.' *FRONTIERS IN IMMUNOLOGY*, 9 p. 1500.

St-Pierre, S., Jiang, W., Roy, P., Champigny, C., LeBlanc, E., Morley, B. J., Hao, J. and Simard, A. R. (2016) 'Nicotinic Acetylcholine Receptors Modulate Bone Marrow-Derived Pro-Inflammatory Monocyte Production and Survival.' *PLoS One*, 11(2) 2016/03/02, p. e0150230.

Starosta, M., Redlicka, J., Brzeziański, M., Niwald, M. and Miller, E. (2016) 'Brain stroke - risk of disability and possibilities of improvment in motor and cognitive functioning.' *Polski merkuriusz lekarski : organ Polskiego Towarzystwa Lekarskiego*, 41(241) p. 39.

Stephan, L. S., Almeida, E. D., Markoski, M. M., Garavaglia, J. and Marcadenti, A. (2017) 'Red Wine, Resveratrol and Atrial Fibrillation.' *Nutrients*, 9(11) p. 1190.

Stephenson, J., Nutma, E., Valk, P. and Amor, S. (2018) 'Inflammation in CNS neurodegenerative diseases.' *Immunology*, 154(2) pp. 204-219.

Strang, F., Scheichl, A., Chen, Y. C., Wang, X., Htun, N. M., Bassler, N., Eisenhardt, S. U., Habersberger, J., et al. (2012) 'Amyloid Plaques Dissociate Pentameric to Monomeric C-Reactive Protein: A Novel Pathomechanism Driving Cortical Inflammation in Alzheimer's Disease?' *Brain Pathology*, 22(3) pp. 337-346.

Su, B., Cheng, J., Yang, J. and Guo, Z. (2001) 'MEKK2 is required for T-cell receptor signals in JNK activation and interleukin-2 gene expression.' *J Biol Chem*, 276(18), May 4, 2001/03/30, pp. 14784-14790.

Sun, Song, D., Wang, M., Chen, K. and Zhang, T. (2017) 'alpha7 nicotinic acetylcholine receptor agonist attenuates the cerebral injury in a rat model of cardiopulmonary bypass by activating the Akt/GSK3beta pathway.' *Mol Med Rep*, 16(6), Dec, 2017/09/26, pp. 7979-7986.

Sun, Zhou, K., Wang, S., Li, P., Chen, S., Lin, G., Zhao, Y. and Wang, T. (2013a) 'Involvement of MAPK/NF-kappaB signaling in the activation of the cholinergic anti-inflammatory pathway in experimental colitis by chronic vagus nerve stimulation.' *PLoS One*, 8(8) 2013/08/13, p. e69424.

Sun, Zhou, K., Wang, S., Li, P., Chen, S., Lin, G., Zhao, Y. and Wang, T. (2013b) 'Involvement of MAPK/NF-κB signaling in the activation of the cholinergic anti-inflammatory pathway in experimental colitis by chronic vagus nerve stimulation.' *PLoS One*, 8(8) 2013/08/13, p. e69424.

Sundström, C., Sundstrom, C., Nilsson, K. and Nilsson, K. (1976) 'Establishment and characterization of a human histiocytic lymphoma cell line (U 937).' *International Journal of Cancer*, 17(5) pp. 565-577.

Swirski and Nahrendorf, M. (2013) 'Leukocyte behavior in atherosclerosis, myocardial infarction, and heart failure.' *Science*, 339(6116), Jan 11, 2013/01/12, pp. 161-166.

Szalai, VanCott, J. L., McGhee, J. R., Volanakis, J. E. and William, H. B., Jr. (2000) 'Human C-Reactive Protein Is Protective against Fatal Salmonella enterica Serovar Typhimurium Infection in Transgenic Mice.' *Infection and Immunity*, 68(10) pp. 5652-5656.

Szalai, van Ginkel, F. W., Dalrymple, S. A., Murray, R., McGhee, J. R. and Volanakis, J. E. (1998a) 'Testosterone and IL-6 Requirements for Human C-Reactive Protein Gene Expression in Transgenic Mice.' *The Journal of Immunology*, 160(11) pp. 5294-5299.

Szalai, van Ginkel, F. W., Dalrymple, S. A., Murray, R., McGhee, J. R. and Volanakis, J. E. (1998b) 'Testosterone and IL-6 requirements for human C-reactive protein gene expression in transgenic mice.' *J Immunol*, 160(11), Jun 1, 1998/05/30, pp. 5294-5299.

Tak, P. P. and Firestein, G. S. (2001) 'NF-kappaB: a key role in inflammatory diseases.' *J Clin Invest*, 107(1), Jan, 2001/01/03, pp. 7-11.

Tanaka, Narazaki, M. and Kishimoto, T. (2014) 'IL-6 in inflammation, immunity, and disease.' *Cold Spring Harb Perspect Biol*, 6(10), Sep 4, 2014/09/06, p. a016295.

Tanaka, Kamanaka, M., Enslen, H., Dong, C., Wysk, M., Davis, R. J. and Flavell, R. A. (2002) 'Differential involvement of p38 mitogen-activated protein kinase kinases MKK3 and MKK6 in T-cell apoptosis.' 3(8), Aug, 2002/08/02, pp. 785-791.

Tanigaki, K., Sundgren, N., Khera, A., Vongpatanasin, W., Mineo, C. and Shaul, P. W. (2015) 'Fcγ Receptors and Ligands and Cardiovascular Disease.' *Circulation Research*, 116(2) pp. 368-384.

Tarawneh, R. and Holtzman, D. M. (2012) 'The clinical problem of symptomatic Alzheimer disease and mild cognitive impairment.' *Cold Spring Harb Perspect Med*, 2(5), May, 2012/05/04, p. a006148.

Tarek, József, L., Potempa, L. A. and Filep, J. n. G. (2004) 'Conformational Rearrangement in C-Reactive Protein Is Required for Proinflammatory Actions on Human Endothelial Cells.' *Circulation (New York, N.Y.)*, 109(16) pp. 2016-2022.

Tarek, Jozsef, L., Potempa, L. A. and Filep, J. G. (2006) 'Loss of pentameric symmetry of C-reactive protein induces interleukin-8 production through peroxynitrite signaling in human neutrophils.' *The FASEB Journal*, 20(5) pp. A1084-A1084.

Tarek, Levente, Hossain, S., John, S. D. C., Potempa, L. A. and Filep, J. n. G. (2002) 'Loss of Pentameric Symmetry of C-reactive Protein Is Associated with Delayed Apoptosis of Human Neutrophils.' *Journal of Biological Chemistry*, 277(43) pp. 40775-40781.

Taylor, K. E. and Van Den Berg, C. W. (2007) 'Structural and functional comparison of native pentameric, denatured monomeric and biotinylated C-reactive protein.' *Immunology*, 120(3) pp. 404-411.

Thiele, J. R., Zeller, J., Bannasch, H., Stark, G. B., Peter, K. and Eisenhardt, S. U. (2015) 'Targeting C-Reactive Protein in Inflammatory Disease by Preventing Conformational Changes.' *MEDIATORS OF INFLAMMATION*, 2015 pp. 1-9.

Thiele, J. R., Habersberger, J., Braig, D., Schmidt, Y., Goerendt, K., Maurer, V., Bannasch, H., Scheichl, A., et al. (2014a) 'Dissociation of Pentameric to Monomeric C-Reactive Protein Localizes and Aggravates Inflammation In Vivo Proof of a Powerful Proinflammatory Mechanism and a New Anti-Inflammatory Strategy.' *CIRCULATION*, 130(1) pp. 35-35.

Thiele, J. R., Habersberger, J., Braig, D., Schmidt, Y., Goerendt, K., Maurer, V., Bannasch, H., Scheichl, A., et al. (2014b) 'Dissociation of pentameric to monomeric C-reactive protein localizes and aggravates inflammation: in vivo proof of a powerful proinflammatory mechanism and a new anti-inflammatory strategy.' *Circulation*, 130(1), Jul 1, 2014/07/02, pp. 35-50.

Thompson, D., Pepys, M. B. and Wood, S. P. (1999) 'The physiological structure of human C-reactive protein and its complex with phosphocholine.' *Structure*, 7(2) pp. 169-177.

Thomsen, M. S. and Mikkelsen, J. D. (2012) 'The α7 nicotinic acetylcholine receptor ligands methyllycaconitine, NS6740 and GTS-21 reduce lipopolysaccharide-induced TNF-α release from microglia.' *Journal of Neuroimmunology*, 251(1) pp. 65-72.

Thuraisingam, Xu, Y. Z., Eadie, K., Heravi, M., Guiot, M.-C., Greemberg, R., Gaestel, M. and Radzioch, D. (2010) 'MAPKAPK-2 Signaling Is Critical for Cutaneous Wound Healing.' *Journal of Investigative Dermatology*, 130(1) pp. 278-286.

Torzewski, M., Rist, C., Mortensen, R. F., Zwaka, T. P., Bienek, M., Waltenberger, J., Koenig, W., Schmitz, G., et al. (2000) 'C-reactive protein in the arterial intima: role of C-reactive protein receptor-dependent monocyte recruitment in atherogenesis.' *Arterioscler Thromb Vasc Biol*, 20(9), Sep, 2000/09/09, pp. 2094-2099.

Tousoulis, Charakida, M. and Stefanadis, C. (2006) 'Endothelial function and inflammation in coronary artery disease.' *Heart*, 92(4), Apr, 2005/09/15, pp. 441-444.

Towbin, H., Staehelin, T. and Gordon, J. (1979) 'Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications.' *Proceedings of the National Academy of Sciences of the United States of America*, 76(9) pp. 4350-4354.

Tracey. (2002) 'The inflammatory reflex.' *Nature*, 420(6917) pp. 853-859.

Tracey. (2007) 'Physiology and immunology of the cholinergic antiinflammatory pathway.' *The Journal of clinical investigation*, 117(2) pp. 289-296.

Tracey. (2009) 'Reflex control of immunity.' *Nature Reviews Immunology*, 9(6) pp. 418-428.

Trial, Potempa LA and ML., E. (2016) 'The Role of C-reactive Protein in Innate and Acquired Inflammation: New Perspectives.' *Inflammation and Cell Signaling*,

Tron, K., Manolov, D. E., Röcker, C., Kächele, M., Torzewski, J. and Nienhaus, G. U. (2008) 'C-reactive protein specifically binds to Fcγ receptor type I on a macrophage-like cell line.' *European Journal of Immunology*, 38(5) pp. 1414-1422.

Tsalamandris, S., Antonopoulos, A. S., Oikonomou, E., Papamikroulis, G. A., Vogiatzi, G., Papaioannou, S., Deftereos, S. and Tousoulis, D. (2019) 'The Role of Inflammation in Diabetes: Current Concepts and Future Perspectives.' *Eur Cardiol*, 14(1), Apr, 2019/05/28, pp. 50-59.

Tsoyi, K., Jang, H. J., Kim, J. W., Chang, H. K., Lee, Y. S., Pae, H.-O., Kim, H. J., Seo, H. G., et al. (2011) 'Stimulation of Alpha7 Nicotinic Acetylcholine Receptor by Nicotine Attenuates Inflammatory Response in Macrophages and Improves Survival in Experimental Model of Sepsis Through Heme Oxygenase-1 Induction.' *Antioxidants & Redox Signaling*, 14(11) pp. 257-2070.

Tucureanu, M. M., Rebleanu, D., Constantinescu, C. A., Deleanu, M., Voicu, G., Butoi, E., Calin, M. and Manduteanu, I. (2018) 'Lipopolysaccharide-induced inflammation in monocytes/macrophages is blocked by liposomal delivery of Gi-protein inhibitor.' *International journal of nanomedicine*, 13 p. 63.

Turu, Slevin, M., Matou, S., West, D., Rodriguez, C., Luque, A., Grau-Olivares, M., Badimon, L., et al. (2008) 'C-reactive protein exerts angiogenic effects on vascular endothelial cells and modulates associated signalling pathways and gene expression.' *BMC Cell Biol*, 9, Sep 2, 2008/09/04, p. 47.

Ulleryd, M., Mjörnstedt, F., Panagaki, D., Yang, L. J., Engevall, K., Gutierrez, S., Nilsson, H., Michaëlsson, E., et al. (2019) 'The Alpha 7 Nicotinic Acetylcholine Receptor (A7nachr) Agonist Az6983 Reduces Murine Atherosclerosis And Inflammatory Cytokines In Human Blood Cells.' *Atherosclerosis*, 287 pp. e101-e101.

Upadhya, D., Ogata, M. and Reneker, L. W. (2013) 'MAPK1 is required for establishing the pattern of cell proliferation and for cell survival during lens development.' *Development (Cambridge)*, 140(7) pp. 1573-1582.

Urra, X., Cervera, A., Obach, V., Climent, N., Planas, A. M. and Chamorro, A. (2009) 'Monocytes are major players in the prognosis and risk of infection after acute stroke.' *Stroke*, 40(4), Apr, 2009/01/24, pp. 1262-1268.

Valones, M. A. A., Guimarães, R. L., Brandão, L. A. C., De Souza, P. R. E., De Albuquerque Tavares Carvalho, A. and Crovela, S. (2009) 'Principles and applications of polymerase chain reaction in medical diagnostic fields: A review.' *Brazilian Journal of Microbiology*, 40(1) pp. 1-11.

van de Winkel, J. G. and Anderson, C. L. (1991) 'Biology of human immunoglobulin G Fc receptors.' *J Leukoc Biol*, 49(5), May, 1991/05/01, pp. 511-524.

van den Berg, Taylor, K. E. and Lang, D. (2004) 'C-Reactive Protein-Induced In Vitro Vasorelaxation Is an Artefact Caused by the Presence of Sodium Azide in Commercial Preparations.' *Arteriosclerosis, Thrombosis, and Vascular Biology*, 24(10) pp. e168-E171.

van der Bruggen, T., Nijenhuis, S., van Raaij, E., Verhoef, J. and van Asbeck, B. S. (1999) 'Lipopolysaccharide-induced tumor necrosis factor alpha production by human monocytes involves the raf-1/MEK1-MEK2/ERK1-ERK2 pathway.' *Infect Immun*, 67(8), Aug, 1999/07/23, pp. 3824-3829.

Van Deventer, S. J. (1997) 'Tumour necrosis factor and Crohn's disease.' *Gut*, 40(4), Apr, 1997/04/01, pp. 443-448.

Van Dyken, S. J. and Locksley, R. M. (2013) 'Interleukin-4- and interleukin-13-mediated alternatively activated macrophages: roles in homeostasis and disease.' *Annu Rev Immunol*, 31 2013/01/10, pp. 317-343.

van Furth, R. and Cohn, Z. A. (1968) 'The origin and kinetics of mononuclear phagocytes.' *The Journal of experimental medicine*, 128(3) pp. 415-435.

VanGilder, R. L., Davidov, D. M., Stinehart, K. R., Huber, J. D., Turner, R. C., Wilson, K. S., Haney, E., Davis, S. M., et al. (2014) 'C-reactive protein and long-term ischemic stroke prognosis.' *JOURNAL OF CLINICAL NEUROSCIENCE*, 21(4) pp. 547-553.

VanGuilder, H. D., Vrana, K. E. and Freeman, W. M. (2008) 'Twenty-five years of quantitative PCR for gene expression analysis.' *BioTechniques*, 44(5) pp. 619-626.

Venkat, P., Chopp, M. and Chen, J. L. (2015) 'Models and mechanisms of vascular dementia.' *EXPERIMENTAL NEUROLOGY*, 272 pp. 97-108.

Venugopal, Devaraj, S. and Jialal, I. (2003) 'C-reactive protein decreases prostacyclin release from human aortic endothelial cells.' *Circulation*, 108(14), Oct 7, 2003/09/25, pp. 1676-1678.

Venugopal, Devaraj, S., Yuhanna, I., Shaul, P. and Jialal, I. (2002) 'Demonstration That C-Reactive Protein Decreases eNOS Expression and Bioactivity in Human Aortic Endothelial Cells.' *Circulation (New York, N.Y.)*, 106(12) pp. 1439-1441.

Verma, S., Szmitko, P. E. and Yeh, E. T. (2004) 'C-reactive protein: structure affects function.' *Circulation*, 109(16), Apr 27, 2004/05/01, pp. 1914-1917.

Vidyasagar, A., Wilson, N. A. and Djamali, A. (2012) 'Heat shock protein 27 (HSP27): biomarker of disease and therapeutic target.' *Fibrogenesis Tissue Repair*, 5(1), May 7, 2012/05/09, p. 7.

Vijayan, M. and Reddy, P. H. (2016) 'Stroke, Vascular Dementia, and Alzheimer's Disease: Molecular Links.' *Journal of Alzheimer's disease : JAD U6 US U7 - Journal Article*, 54(2) p. 427.

Vijayan, M., Kumar, S., Bhatti, J. S. and Reddy, P. H. (2017) 'Molecular Links and Biomarkers of Stroke, Vascular Dementia, and Alzheimer's Disease.' *Prog Mol Biol Transl Sci*, 146 2017/03/04, pp. 95-126.

Visser, M., Bouter, L. M., McQuillan, G. M., Wener, M. H. and Harris, T. B. (1999) 'Elevated C-reactive protein levels in overweight and obese adults.' *JAMA*, 282(22), Dec 8, 1999/12/11, pp. 2131-2135.

Vogel, C. F., Garcia, J., Wu, D., Mitchell, D. C., Zhang, Y., Kado, N. Y., Wong, P., Trujillo, D. A., et al. (2012) 'Activation of inflammatory responses in human U937 macrophages by particulate matter collected from dairy farms: an in vitro expression analysis of pro-inflammatory markers.' *Environ Health*, 11, Mar 28, 2012/03/29, p. 17.

Vogel, R. A., Corretti, M. C. and Gellman, J. (1998) 'Cholesterol, cholesterol lowering, and endothelial function.' *Progress in Cardiovascular Diseases*, 41(2) pp. 117-136.

Vogelpoel, L. T., Baeten, D. L., de Jong, E. C. and den Dunnen, J. (2015) 'Control of cytokine production by human fc gamma receptors: implications for pathogen defense and autoimmunity.' *Front Immunol*, 6 2015/03/12, p. 79.

Volanakis. (2001) Human C-reactive protein: expression, structure, and function. Vol. 38, pp. 189-197. OXFORD: Elsevier Ltd.

Volanakis and Kaplan, M. H. (1971) 'Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide.' *Proc Soc Exp Biol Med*, 136(2), Feb, 1971/02/01, pp. 612-614.

Wada and Penninger, J. M. (2004) 'Mitogen-activated protein kinases in apoptosis regulation.' *Oncogene*, 23(16), Apr 12, 2004/04/13, pp. 2838-2849.

Waldburger, J.-M., Boyle, D. L., Pavlov, V. A., Tracey, K. J. and Firestein, G. S. (2008) 'Acetylcholine regulation of synoviocyte cytokine expression by the alpha7 nicotinic receptor.' *Arthritis and rheumatism*, 58(11) p. 3439.

Wallquist, C., Paulson, J. M., Hylander, B., Lundahl, J. and Jacobson, S. H. (2013) 'Increased Accumulation of CD16+ Monocytes at Local Sites of Inflammation in Patients with Chronic Kidney Disease.' *Scandinavian journal of immunology*, 78(6) pp. 538-544.

Wang and Harris, D. C. (2011) 'Macrophages in renal disease.' *J Am Soc Nephrol*, 22(1), Jan, 2011/01/07, pp. 21-27.

Wang, Tang, X. N. and Yenari, M. A. (2006) 'The inflammatory response in stroke.' *Journal of Neuroimmunology*, 184(1) pp. 53-68.

Wang, Destrument, A. and Tournier, C. (2007) 'Physiological roles of MKK4 and MKK7: insights from animal models.' *Biochim Biophys Acta*, 1773(8), Aug, 2006/12/13, pp. 1349-1357.

Wang, Liang, H. and Zen, K. (2014) 'Molecular mechanisms that influence the macrophage m1-m2 polarization balance.' *Frontiers in immunology*, 5 p. 614.

Wang, Yan, L., Wesley, R. A. and Danner, R. L. (1997) 'Nitric Oxide Increases Tumor Necrosis Factor Production in Differentiated U937 Cells by Decreasing Cyclic AMP.' *Journal of Biological Chemistry*, 272(9) pp. 5959-5965.

Wang, Yatsuya, H., Tamakoshi, K., Uemura, M., Li, Y., Wada, K., Yamashita, K., Kawaguchi, L., et al. (2013) 'Positive association between high-sensitivity C-reactive protein and incidence of type 2 diabetes mellitus in Japanese workers: 6-year follow-up: hs-CRP and Type 2 Diabetes Incidence.' *Diabetes/metabolism research and reviews*, 29(5) pp. 398-405.

Wang, Yu, M., Ochani, M., Amella, C. A., Tanovic, M., Susarla, S., Li, J. H., Wang, H. C., et al. (2003) 'Nicotinic acetylcholine receptor alpha 7 subunit is an essential regulator of inflammation.' *NATURE*, 421(6921) pp. 384-388.

Wang, Li, Wang, C. D., Xiao, F., Zhu, J. F., Shen, C., Zuo, B., Cui, Y. M., et al. (2017) 'Anti-inflammatory and anti-osteoarthritis effects of tectorigenin.' *Biol Open*, 6(8), Aug 15, 2017/06/24, pp. 1130-1136.

Watanabe, E., Miyake, S. and Yogo, Y. (2013) 'Review of enzyme-linked immunosorbent assays (ELISAs) for analyses of neonicotinoid insecticides in agro-environments.' *Journal of Agricultural and Food Chemistry*, 61(51) pp. 12459-12472.

Wattananit, S., Tornero, D., Graubardt, N., Memanishvili, T., Monni, E., Tatarishvili, J., Miskinyte, G., Ge, R., et al. (2016) 'Monocyte-derived macrophages contribute to spontaneous long-term functional recovery after stroke in mice.' *Journal of Neuroscience*, 36(15) pp. 4182-4195.

Wei, X., Zhang, Y., Li, C., Ai, K., Li, K., Li, H. and Yang, J. (2020) 'The evolutionarily conserved MAPK/Erk signaling promotes ancestral T-cell immunity in fish via c-Myc-mediated glycolysis.' *J Biol Chem*, 295(10), Mar 6, 2020/01/31, pp. 3000-3016.

Weinstein, G., Beiser, A. S., Decarli, C., Au, R., Wolf, P. A. and Seshadri, S. (2013) 'Brain imaging and cognitive predictors of stroke and Alzheimer disease in the Framingham Heart Study.' *Stroke*, 44(10) pp. 2787-2794.

Weiss, U. (2008) 'Inflammation.' *Nature*, 454(7203) pp. 427-427.

Wellbrock, Karasarides, M. and Marais, R. (2004) 'The RAF proteins take centre stage.' *Nat Rev Mol Cell Biol*, 5(11), Nov, 2004/11/03, pp. 875-885.

Wentworth, Naselli, G., Brown, W. A., Doyle, L., Phipson, B., Smyth, G. K., Wabitsch, M., O'Brien, P. E., et al. (2010) 'Pro-inflammatory CD11c+CD206+ adipose tissue macrophages are associated with insulin resistance in human obesity.' *Diabetes*, 59(7) pp. 1648-1656.

Whitmarsh, A. J. and Davis, R. J. (2007) 'Role of mitogen-activated protein kinase kinase 4 in cancer.' *Oncogene*, 26(22), May 14, 2007/05/15, pp. 3172-3184.

Williams, R. D., Moran, J. A., Fryer, A. A., Littlejohn, J. R., Williams, H. M., Greenhough, T. J. and Shrive, A. K. (2020) 'Monomeric C-Reactive Protein in Serum With Markedly Elevated CRP Levels Shares Common Calcium-Dependent Ligand Binding Properties With an in vitro Dissociated Form of C-Reactive Protein.' *Front Immunol*, 11 2020/03/03, p. 115.

Willis, C. L., Meske, D. S. and Davis, T. P. (2010) 'Protein kinase C activation modulates reversible increase in cortical blood-brain barrier permeability and tight junction protein expression during hypoxia and posthypoxic reoxygenation.' *J Cereb Blood Flow Metab*, 30(11), Nov, 2010/08/12, pp. 1847-1859.

Winbeck, Poppert, H., Etgen, T., Conrad, B. and Sander, D. (2002) 'Prognostic relevance of early serial C-reactive protein measurements after first ischemic stroke.' *Stroke*, 33(10), Oct, 2002/10/05, pp. 2459-2464.

Woollard, K. J. and Geissmann, F. (2010) 'Monocytes in atherosclerosis: subsets and functions.' *Nat Rev Cardiol*, 7(2), Feb, 2010/01/13, pp. 77-86.

Wortzel, I. and Seger, R. (2011) 'The ERK Cascade: Distinct Functions within Various Subcellular Organelles.' *Genes Cancer*, 2(3), Mar, 2011/07/23, pp. 195-209.

Wouters, K., Gaens, K., Bijnen, M., Verboven, K., Jocken, J., Wetzels, S., Wijnands, E., Hansen, D., et al. (2017) 'Circulating classical monocytes are associated with CD11c(+) macrophages in human visceral adipose tissue.' *Sci Rep*, 7, Feb 15, 2017/02/16, p. 42665.

Wu, Li, L. and Su, X. (2014) 'Vagus Nerve through α7 nAChR Modulates Lung Infection and Inflammation: Models, Cells, and Signals.' *BioMed research international*, 2014 pp. 283525-283520.

Wu, Potempa, L. A., El Kebir, D. and Filep, J. G. (2015) 'C-reactive protein and inflammation: conformational changes affect function.' *BIOLOGICAL CHEMISTRY*, 396(11) pp. 1181-1197.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. and Greenberg, M. E. (1995) 'Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis.' *Science*, 270(5240), Nov 24, 1995/11/24, pp. 1326-1331.

Xiao, Liu, Y. and Wang, N. (2014) 'New paradigms in inflammatory signaling in vascular endothelial cells.' *Am J Physiol Heart Circ Physiol*, 306(3), Feb, 2013/11/29, pp. H317-325.

Xiao, Liu, C., Tu, Z., Xu, Q., Chen, S., Zhang, Y., Wang, X., Zhang, J., et al. (2020) 'Activation of the NF- κ B and MAPK Signaling Pathways Contributes to the Inflammatory Responses, but Not Cell Injury, in IPEC-1 Cells Challenged with Hydrogen Peroxide.' *Oxidative medicine and cellular longevity*, 2020 pp. 1-14.

Xie, C., Liu, C., Wu, B., Lin, Y., Ma, T., Xiong, H., Wang, Q., Li, Z., et al. (2016) 'Effects of IRF1 and IFN-beta interaction on the M1 polarization of macrophages and its antitumor function.' *Int J Mol Med*, 38(1), Jul, 2016/05/14, pp. 148-160.

Xiong, H. and Pamer, E. G. (2014) 'Monocytes and infection: Modulator, messenger and effector.' *Immunobiology*, 220(2) pp. 210-214.

Xu, Shi, Q., Mo, Y., Wu, L., Gu, J. and Xu, Y. (2019) 'Downregulation of α7 nicotinic acetylcholine receptors in peripheral blood monocytes is associated with enhanced inflammation in preeclampsia.' *BMC pregnancy and childbirth*, 19(1) pp. 188-189.

Xu, W., Zhao, X., Daha, M. R. and van Kooten, C. (2013) 'Reversible differentiation of pro- and anti-inflammatory macrophages.' *Mol Immunol*, 53(3), Mar, 2012/09/05, pp. 179-186.

Yaghi, S., Prabhakaran, S., Khatri, P. and Liebeskind, D. S. (2019) 'Intracranial Atherosclerotic Disease.' *Stroke*, 50(5), May, 2019/04/23, pp. 1286-1293.

Yamada and Ichinose, M. (2018) 'The Cholinergic Pathways in Inflammation: A Potential Pharmacotherapeutic Target for COPD.' *Front Pharmacol*, 9 2018/12/19, p. 1426.

Yamadera, S., Nakamura, Y., Inagaki, M., Kenmotsu, S., Nohara, T., Sato, N., Oguchi, T., Tsuji, M., et al. (2018) 'Linagliptin inhibits lipopolysaccharide-induced inflammation in human U937 monocytes.' *Inflamm Regen*, 38 2018/08/29, p. 13.

Yamagata, K., Kitazawa, T., Shinoda, M., Tagawa, C., Chino, M. and Matsufuji, H. (2010) 'Stroke status evoked adhesion molecule genetic alterations in astrocytes isolated from stroke-prone spontaneously hypertensive rats and the apigenin inhibition of their expression.' *Stroke research and treatment*, 2010 pp. 1-11.

Yan, X. X., Toth, Z., Schultz, L., Ribak, C. E. and Baram, T. Z. (1998) 'Corticotropin-releasing hormone (CRH)-containing neurons in the immature rat hippocampal formation: light and electron microscopic features and colocalization with glutamate decarboxylase and parvalbumin.' *Hippocampus*, 8(3) 1998/07/14, pp. 231-243.

Yang, Yin, Y. and Wang, H. (2008) 'VASCULAR INFLAMMATION AND ATHEROGENESIS ARE ACTIVATED VIA RECEPTORS FOR PAMPs AND SUPPRESSED BY REGULATORY T CELLS.' *Drug Discov Today Ther Strateg*, 5(2) 2008/01/01, pp. 125-142.

Yang, Wang, J., Brand, D. D. and Zheng, S. G. (2018) 'Role of TNF-TNF Receptor 2 Signal in Regulatory T Cells and Its Therapeutic Implications.' *Front Immunol*, 9 2018/05/05, p. 784.

Yang, Zhang, L., Yu, C., Yang, X.-F. and Wang, H. (2014a) 'Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases.' *Biomarker research*, 2(1) p. 1.

Yang, Lee, K. T., Park, H. J., Kim, T. J., Choi, Y. S., Shih Ie, M. and Choi, J. H. (2012) 'Tectorigenin sensitizes paclitaxel-resistant human ovarian cancer cells through downregulation of the Akt and NFκB pathway.' *Carcinogenesis*, 33(12), Dec, 2012/10/03, pp. 2488-2498.

Yang, Kim, S. C., Yu, T., Yi, Y.-S., Rhee, M. H., Sung, G.-H., Yoo, B. C. and Cho, J. Y. (2014b) 'Functional roles of p38 mitogen-activated protein kinase in macrophage-mediated inflammatory responses.' *Mediators of inflammation*, 2014 pp. 352371-352313.

Yang, Wezeman, M., Zhang, X., Lin, P., Wang, M., Qian, J., Wan, B., Kwak, L. W., et al. (2007) 'Human C-Reactive Protein Binds Activating Fcγ Receptors and Protects Myeloma Tumor Cells from Apoptosis.' *Cancer Cell*, 12(3) pp. 252-265.

Yao, Y., Xu, X. H. and Jin, L. (2019) 'Macrophage Polarization in Physiological and Pathological Pregnancy.' *Front Immunol*, 10 2019/05/01, p. 792.

Yaron, G., Brill, A., Dashevsky, O., Yosef-Levi, I. M., Grad, E., Danenberg, H. D. and Varon, D. (2006) 'C-reactive protein promotes platelet adhesion to endothelial cells: a potential pathway in atherothrombosis.' *Br J Haematol*, 134(4), Aug, 2006/07/11, pp. 426-431.

Yasojima, Schwab, C., McGeer, E. G. and McGeer, P. L. (2000) 'Human neurons generate C-reactive protein and amyloid P: upregulation in Alzheimer's disease.' *Brain Res*, 887(1), Dec 22, 2001/01/03, pp. 80-89.

Yasojima, K., Schwab, C., McGeer, E. G. and McGeer, P. L. (2000) 'Human neurons generate C-reactive protein and amyloid P: Upregulation in Alzheimer's disease.' *Brain Research*, 887(1) pp. 80-89.

Yasojima, K., Schwab, C., McGeer, E. G. and McGeer, P. L. (2001) 'Generation of C-reactive protein and complement components in atherosclerotic plaques.' *Am J Pathol*, 158(3), Mar, 2001/03/10, pp. 1039-1051.

Yenari, M. A. and Han, H. S. (2006) 'Influence of hypothermia on post-ischemic inflammation: Role of nuclear factor kappa B (NFκB).' *Neurochemistry International*, 49(2) pp. 164-169.

Yong and Cao, H. (2020) 'Monomeric C-reactive protein affects cell injury and apoptosis through activation of p38 MAPK in human coronary artery endothelial cells.' *Bosnian journal of basic medical sciences*,

Yoon, S. and Seger, R. (2006) 'The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions.' *Growth Factors*, 24(1), Mar, 2006/01/06, pp. 21-44.

Yoshikawa, H., Kurokawa, M., Ozaki, N., Nara, K., Atou, K., Takada, E., Kamochi, H. and Suzuki, N. (2006a) 'Nicotine inhibits the production of proinflammatory mediators in human monocytes by suppression of I-kappaB phosphorylation and nuclear factor-kappaB transcriptional activity through nicotinic acetylcholine receptor alpha7.' *Clinical and experimental immunology*, 146(1) p. 116.

Yoshikawa, H., Kurokawa, M., Ozaki, N., Nara, K., Atou, K., Takada, E., Kamochi, H. and Suzuki, N. (2006b) 'Nicotine inhibits the production of proinflammatory mediators in human monocytes by suppression of I-kappaB phosphorylation and nuclear factor-kappaB transcriptional activity through nicotinic acetylcholine receptor alpha7.' *Clin Exp Immunol*, 146(1), Oct, 2006/09/14, pp. 116-123.

Yoshimura, A., Ohnishi, S., Orito, C., Kawahara, Y., Takasaki, H., Takeda, H., Sakamoto, N. and Hashino, S. (2015) 'Association of peripheral total and differential leukocyte counts with obesity-related complications in young adults.' *Obes Facts*, 8(1) 2015/03/15, pp. 1-16.

You, C. J., Liu, D., Liu, L. L. and Li, G. Z. (2018) 'Correlation between acute stroke-induced white matter lesions and insulin resistance.' *MEDICINE*, 97(11) p. e9860.

Yu, Y.-R. A., O'Koren, E. G., Hotten, D. F., Kan, M. J., Kopin, D., Nelson, E. R., Que, L. and Gunn, M. D. (2016) 'A Protocol for the Comprehensive Flow Cytometric Analysis of Immune Cells in Normal and Inflamed Murine Non-Lymphoid Tissues.' *PloS one*, 11(3) p. e0150606.

Yuan and Silberstein, S. D. (2016) 'Vagus Nerve and Vagus Nerve Stimulation, a Comprehensive Review: Part I.' *Headache: The Journal of Head and Face Pain*, 56(1) pp. 71-78.

Zarruk, J. G., Greenhalgh, A. D. and David, S. (2018) 'Microglia and macrophages differ in their inflammatory profile after permanent brain ischemia.' *Experimental Neurology*, 301(Pt B) pp. 120-132.

Zarubin and Han, J. (2005) 'Activation and signaling of the p38 MAP kinase pathway.' *Cell Research*, 15(1) pp. 11-18.

Zeller, I. and Srivastava, S. (2014) 'Macrophage functions in atherosclerosis.' *Circ Res*, 115(12), Dec 5, 2014/12/06, pp. e83-85.

Zelová, H. and Hošek, J. (2013) 'TNF-α signalling and inflammation: interactions between old acquaintances.' *Inflammation research*, 62(7) pp. 641-651.

Zeng, Yuan, S., Shen, J., Wu, M., Pan, L. and Kong, X. (2018) 'Suppression of human breast cancer cells by tectorigenin through downregulation of matrix metalloproteinases and MAPK signaling in vitro.' *Mol Med Rep*, 17(3), Mar, 2018/01/24, pp. 3935-3943.

Zhang and An, J. (2007) 'Cytokines, inflammation, and pain.' *International anesthesiology clinics*, 45(2) p. 27.

Zhang, Wang, S. and Fang, G. (2011) 'Applications and recent developments of multi-analyte simultaneous analysis by enzyme-linked immunosorbent assays.' *Journal of Immunological Methods*, 368(1-2) pp. 1-23.

Zhang, Sun, M., Samols, D. and Kushner, I. (1996) 'STAT3 participates in transcriptional activation of the C-reactive protein gene by interleukin-6.' *J Biol Chem*, 271(16), Apr 19, 1996/04/19, pp. 9503-9509.

Zhang, Xia, Y., Jiang, W., Wang, C., Han, B. and Hao, J. (2016) 'Determination of non-neuronal acetylcholine in human peripheral blood mononuclear cells by use of hydrophilic interaction ultra-performance liquid chromatography–tandem mass spectrometry.' *Journal of Chromatography B*, 1022 pp. 265-273.

Zhang, He, L., Yue, S., Huang, Q., Zhang, Y. and Yang, J. (2017a) 'Characterization and evaluation of a self-microemulsifying drug delivery system containing tectorigenin, an isoflavone with low aqueous solubility and poor permeability.' *Drug Deliv*, 24(1), Nov, 2017/03/12, pp. 632-640.

Zhang, Li, H. Y., Li, W., Shen, Z. Y., Wang, Y. D., Ji, S. R. and Wu, Y. (2018) 'An ELISA Assay for Quantifying Monomeric C-Reactive Protein in Plasma.' *FRONTIERS IN IMMUNOLOGY*, 9

Zhang, Zhao, J., Wang, R., Jiang, M., Ye, Q., Smith, A. D., Chen, J. and Shi, Y. (2019) 'Macrophages reprogram after ischemic stroke and promote efferocytosis and inflammation resolution in the mouse brain.' *CNS Neurosci Ther*, 25(12), Dec, 2019/11/08, pp. 1329-1342.

Zhang, Yuan, L., Zhang, Q. R., Gao, Y., Liu, G. H., Xiu, M., Wei, X., Wang, Z., et al. (2015) 'Resveratrol attenuates hypoxia-induced neurotoxicity through inhibiting microglial activation.' *INTERNATIONAL IMMUNOPHARMACOLOGY*, 28(1) pp. 578-587.

Zhang, Cao, M., He, Y., Liu, Y., Zhang, G., Yang, C., Du, Y., Xu, J., et al. (2017b) 'Increased circulating M2-like monocytes in patients with breast cancer.' *Tumour Biol*, 39(6), Jun, 2017/06/24, p. 1010428317711571.

Zhang, Y. and Yang, W.-X. (2016) 'Tight junction between endothelial cells: the interaction between nanoparticles and blood vessels.' *Beilstein journal of nanotechnology*, 7(1) pp. 675-684.

Zhao, Y. L., Tian, P. X., Han, F., Zheng, J., Xia, X. X., Xue, W. J., Ding, X. M. and Ding, C. G. (2017) 'Comparison of the characteristics of macrophages derived from murine spleen, peritoneal cavity, and bone marrow.' *J Zhejiang Univ Sci B*, 18(12), Dec., 2017/12/06, pp. 1055-1063.

Zhou, Xu, M. J. and Gao, B. (2016) 'Hepatocytes: a key cell type for innate immunity.' *Cell Mol Immunol*, 13(3), May, 2015/12/22, pp. 301-315.

Zhou, Hughes, A. K., Grady, S. C. and Fang, L. (2018) 'Physical activity and chronic diseases among older people in a mid-size city in China: a longitudinal investigation of bipolar effects.' *BMC PUBLIC HEALTH*, 18(1) pp. 1-15.

Zhou, Ma, Y., Sugiura, R., Kobayashi, D., Suzuki, M., Deng, L. and Kuno, T. (2010) 'MAP kinase kinase kinase (MAPKKK)-dependent and -independent activation of Sty1 stress MAPK in fission yeast.' *J Biol Chem*, 285(43), Oct 22, 2010/08/24, pp. 32818-32823.

Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D. N., Leenen, P. J. M., Liu, Y.-J., et al. (2010) 'Nomenclature of monocytes and dendritic cells in blood.' *Blood*, 116(16) pp. e74-e80.

Ziv-Baran, T., Shenhar-Tsarfaty, S., Etz-Hadar, I., Goldiner, I., Gottreich, A., Alcalay, Y., Zeltser, D., Shapira, I., et al. (2017) 'The ability of the wide range CRP assay to classify individuals with low grade inflammation into cardiovascular risk groups.' *Clinica chimica acta*, 471 pp. 185-190.

Zou, D., Luo, M., Han, Z., Zhan, L., Zhu, W., Kang, S., Bao, C., Li, Z., et al. (2017) 'Activation of Alpha-7 Nicotinic Acetylcholine Receptor Reduces Brain Edema in Mice with Ischemic Stroke and Bone Fracture.' *Molecular Neurobiology*, 54(10) pp. 8278-8286.

Zwick, R. K., Guerrero-Juarez, C. F., Horsley, V. and Plikus, M. V. (2018) 'Anatomical, Physiological, and Functional Diversity of Adipose Tissue.' *Cell Metab*, 27(1), Jan 9, 2018/01/11, pp. 68-83.